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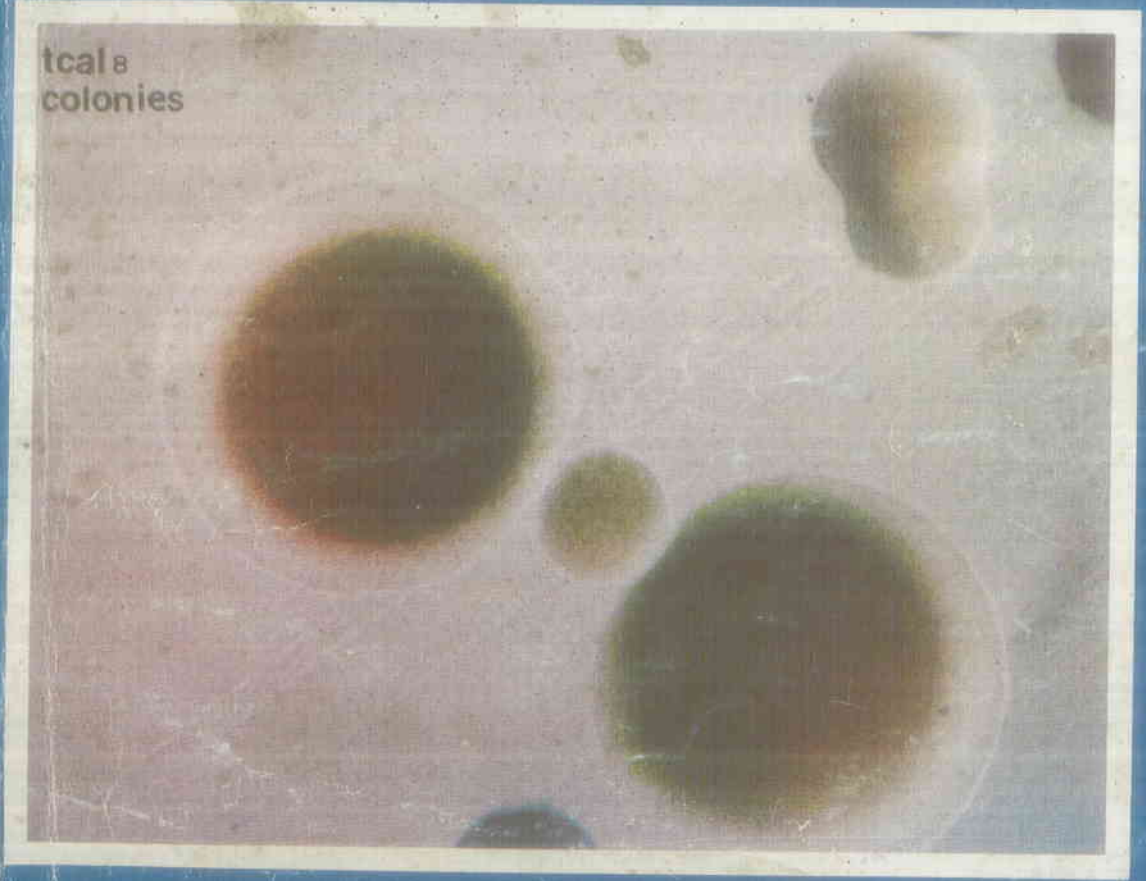

Colonies of *Saccharum spontaneum* L. ('Talahib') isolate in nutrient agar

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NODULATION, BIOMASS PRODUCTION AND NITROGEN FIXATION OF *SAMANEA SAMAN* (JACQ.) MERR. ("ACACIA")*

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ABSTRACT

Inoculation of *Samanea saman* (Jacq.) Merr. ("acacia") seeds with rhizobia increased significantly the total nitrogen uptake and Δ nitrogen fixation of the plants under screenhouse conditions. In the field, rhizobial inoculation or phosphorus pelleting also increased significantly the total biomass production of acacia. A combination of rhizobial inoculation and phosphorus pelleting improved further the biomass production.

INTRODUCTION

One of the leguminous species being recommended for reforestation is *Samanea saman* (Jacq.) Merr. syn. *Enterolobium saman* (Jacq.) Prain commonly called "acacia" or rain tree. In 11 of 12 regions in the Philippines, *S. saman* is included among those species belonging to the first priority for lowland (1200 meters and below) reforestation (PCARRD, 1982). It is recommended primarily not only for timber production and beautification but also for regeneration of degraded uplands since it has a good potential for fixing atmospheric nitrogen (Manguiat, 1982). It thrives well on poor soils, germinates easily, grows quickly, produces seeds plentifully, and suppresses weeds (NAS, 1979). It can serve also as a good component of an agroforestry system since it produces nutritious pods which can be used as feed for livestock.

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Very limited research work has been done locally on nodulation and nitrogen fixation of *S. saman* (Quiniones, 1983). It is being studied by foresters in the Philippines not for its nitrogen-fixing potential but for premium timber production (Halos, 1982). Maun (1978) pointed out that the wood has been particularly preferred by the wood carving industry resulting in a depletion of supply due to over-exploitation. It is no wonder therefore that *S. saman* is considered now as one of the vanishing species among leguminous trees.

Illegal logging and "kaingin" (slash and burn) farming have led to a wanton destruction of our forests resulting in large tracts of denuded/degraded grasslands. The reforestation/ regeneration of these grassland areas is very expensive. Based on the experience of the National Irrigation Administration (NIA), the initial total cost of planting per hectare per year is about ₱3,000 (PCARRD, 1982). This cost includes a substantial amount for fertilization because denuded grassland areas are generally deficient in nitrogen and phosphorus. Utilization of leguminous trees such as acacia as reforestation/regeneration species should be able to contribute considerably to the reduction in fertilization cost because acacia if nodulated by the appropriate rhizobia may be capable of providing its own nitrogen needs through fixation of atmospheric nitrogen. Its contribution to the regeneration of grassland ecosystems may be appreciated better by considering that during summer time it sheds its leaves and pods. Subsequently, the leaves and pods decompose and eventually enrich the soil with organic nitrogen which could be made available to the accompanying or succeeding non-leguminous agroforestry crops through mineralization.

A key to the successful utilization of *S. saman* as a reforestation/regeneration species in grassland areas could be rhizobial inoculation. The present study was designed, therefore, to evaluate the nodulation, biomass production, and nitrogen fixation of *S. saman* with and without rhizobial inoculation under both screenhouse and field conditions.

MATERIALS AND METHODS

Screenhouse experiment. Bulk surface soil samples (0-15 cm) of Annam silty clay loam, an acidic soil with a pH of 5.2 and an organic matter content of 7.47% were collected from a grassland area in Pantabangan, Nueva Ecija. The bulk samples were prepared for sowing as decribed in another paper (Manguiat and Mendoza, 1985) except that only 2 kg of soil was utilized per pot.

Two levels of treatment/isolate (uninoculated and BEs-3) and nine sampling times (2, 4, 6, 8, 10, 12, 14, 16, and 18 weeks after sowing) were used. The experimental design was a 2 x 9 factorial in a completely randomized design (CRD) with three replications. The seeds were scarified with concentrated sulfuric acid, washed thoroughly with water, inoculated with rhizobia, pelleted with phosphorus, and sown in the same manner as described by Manguiat and Mendoza (1985). A pelleting ratio of 1:1 by weight (seed: superphosphate) was used.

The treated and untreated seeds were sown into each pot. Two weeks after sowing, thinning was done and three plants in each pot were maintained until sampling time. At

every sampling time (weeks after sowing), data on nodulation (nodule number and nodule dry weight), and biomass production (root weight, shoot weight and total biomass) were gathered. Nitrogen uptake, total nitrogen and Δ nitrogen fixation were computed as described in another paper (Manguiat and Mendoza, 1985).

The data on nitrogen and Δ nitrogen fixed were analyzed statistically and comparison of means was made by using Duncan's Multiple Range Test (DMRT) at 5% significance level. Regression analyses were performed based on the data on nodulation and biomass production.

Field experiment. The field experiment was conducted in a grassland area at the UPLB-BIOTECH-PESAM Experiment Station in Puting Lupa, Calamba, Laguna. The soil in the experimental area was Macolod silt loam (pH = 5.7 and organic matter content = 6.56%). The experimental area had an average slope of 38%. The experiment was laid out using a randomized complete block design (RCBD) with four replications. Six treatment combinations constituted out of 3 levels of treatment/isolate (uninoculated, BEs-3, and BEs-4) and 2 levels of phosphorus pelleting (O-P and P-pelleted) were tested. BEs-3 and BEs-4 were isolates of rhizobia coming from the root nodule of *S. saman.* These isolates have been studied previously for their infectiveness and effectiveness under growth chamber and screenhouse conditions (Manguiat *et al,* 1984). The transmission electron micrographs of isolates BEs-3 and BEs-4 are shown in Figures 1 and 2, respectively. Figure 3 shows the scanning electron micrographs of bacteroids inside the root nodule of acacia while Figure 4 shows the scanning electron micrographs of both longitudinal and cross sections of acacia nodule.

The area was cleared before setting up the experiment by burning the vegetation while still standing, then cutting at the base before hauling out of the experimental area. Plots with an area of 24 m^2 (6 m x 4 m) each were laid out. Twenty four (24) holes per plot were dug with a distancing of 1 m. Each hole had an area of approximately 400 cm^2 (20 cm x 20 cm) and a depth of about 15 cm.

The seeds of *S. saman* were prepared as in the screenhouse experiment. The treated and untreated seeds were sown respectively on their assigned plots. Ten seeds were sown per hole and they were covered with loose soil to ensure germination. Thinning to three plants per hole was done at 4 weeks after sowing and the plants were grown for 8 months. Weeding was done whenever needed during the growing period.

After 8 months, 4 representative plants were taken randomly from each plot. Data on nodulation, biomass production, and Δ nitrogen fixation were collected. The analysis of plant tissue for total nitrogen, calculation for the nitrogen uptake, and estimation of the magnitude of Δ nitrogen fixation were done following similar procedures as in the screenhouse experiment.

The data were analyzed statistically and for the comparison of means, the Duncan's Multiple Range Test (DMRT) at 10% significance level was chosen due to wide variations within plots and among plots in the experimental area.

RESULTS AND DISCUSSION

A. Screenhouse Experiment

Nodulation pattern. The nodulation of *S. saman* under screenhouse conditions was monitored by counting and weighing at bi-weekly intervals the nodules formed after sowing. A linear nodulation pattern was observed with sampling time as indicated by total nodule number (Fig. 5). The results revealed that the total nodule number of uninoculated plants increased more rapidly than that of the plants inoculated with *Rhizobium* sp. isolate BEs-3. The difference in increase was reflected by the slopes of the regression lines. This observation may be explained by considering that *S. saman* forms nodules in clumps particularly when the seeds were inoculated with rhizobia. Since one clump is counted as one nodule, the more clumps there are, the less the number of nodules formed.

Based on the observed values, no nodules were formed at two weeks after sowing whether or not acacia was inoculated. Apparently, nodule formation started anytime between two and four weeks after sowing. In the case of field legumes, nodulation has been observed to begin at three weeks after planting. It seems that the initiation of nodulation occurs at approximately the same time whether field legumes or tree legumes are involved.

Corby (1971) studied the types of leguminous nodules and cited that *S. saman* forms elongated nodules with branching. In the present experiment, both globose and elongate types of nodules were observed; in many cases, the nodules occur in clumps instead of singly as in field legumes. Consequently, nodule number may not increase with time but a corresponding increase in nodule biomass may be obtained. This statement is supported by the data in Fig. 6 whereby the nodule dry weights of the inoculated plants were somewhat higher than those of the uninoculated control inspite of their lower nodule number as depicted in Fig. 5. These results imply that for tree legumes which form nodules in clumps, nodule biomass is a better parameter than nodule number for assessing the effects of rhizobial inoculation.

Biomass production and nitrogen fixation. Regression analysis revealed that the increase in root weight of acacia with sampling time was exponential (Fig. 7). The root weights of the inoculated plants were slightly higher than those of the uninoculated. Similar to root weight, the increase in the shoot biomass of acacia with time followed a curvilinear model (Fig. 8). The initial increase was very slow but starting from 10 weeks after sowing, a more pronounced influence of rhizobial inoculation was observed. After 18 weeks, an increment of about 9% was obtained suggesting that the effect of inoculation may have been expressed better if the plants were grown for a longer period. The trend for total biomass production was very similar to that of shoot biomass (Fig. 9). These results suggest further that in cases of screenhouse experiments, *S. saman* must be grown for at least 10-12 weeks in order to demonstrate the effects of inoculation treatments.

Inoculation with *Rhizobium* sp. isolate BEs-3 increased significantly the total nitrogen uptake and total Δ nitrogen fixation of acacia (Table 1). Likewise, there was a significant increase in total nitrogen uptake with sampling time. In order to confirm that

S. saman is indeed fixing atmospheric nitrogen, the acetylene-reducing activity of the root system of similarly grown acacia plants was determined using a portable gas chromatograph with similar features as described by Holfeld *et al.* (1979). The regression of digital display (ethylene peak) with incubation time was linear up to one hour (Fig. 10). A linear trend was also obtained for the acetylene-reducing activity (ARA) of *S. saman* with incubation time thereby producing approximately 19 μmoles C_2H_4/g nodule fresh weight/h (Fig. 11). Since ARA is an indirect measure of nitrogen fixation, these results confirm that *S. saman* is indeed fixing atmospheric nitrogen.

B. Field Experiment

Nodulation under field conditions. Eight months after sowing, neither rhizobial inoculation nor phosphorus pelleting affected the nodule number in the primary and lateral roots (Table 2). Nevertheless, by combining the nodule number in the primary + lateral roots, it was noted that *Rhizobium* sp. isolate BEs-4 produced more nodules. In contrast with the effect on nodule number, both rhizobial inoculation and phosphorus pelleting increased significantly the nodule dry weights (Table 3). The effect of rhizobial inoculation on nodule dry weights was enhanced significantly by phosphorus pelleting (Table 4). These results provide additional evidence that nodule biomass is better than nodule number as a measure of treatment effects on the nodulation of *S. saman.* This observation is consistent with the results obtained earlier under screenhouse conditions; hence, in evaluating nodulation of tree legumes which form nodules in clumps, it is no longer necessary to go through a very tedious process of counting the nodules.

Biomass production and nitrogen fixation in the field. Root weight, shoot weight, and total biomass of *S. saman* were improved remarkably by either rhizobial inoculation or phosphorus pelleting (Table 5). Total biomass production was increased by 184-229% by rhizobial inoculation whereas phosphorus pelleting effected an increase of 277%. Upon combining rhizobial inoculation and phosphorus pelleting, the increase in total biomass production based on the uninoculated and non-pelleted control ranged from 579-673% (Table 6). Significant positive interaction effects between rhizobial inoculation and phosphorus pelleting were also obtained on root weight and shoot weight. Similar interaction effects were reported on *Albizia procera* or "akleng parang" (Manguiat and Mendoza, 1985).

The findings in this study clearly indicate that rhizobial inoculation alone is not sufficient to optimize biomass production of *S. saman* in grassland soils. Adequate amount of phosphorus is also needed and it may be supplied through pelleting. However, the possibility of providing phosphorus through mycorrhizal inoculation should be explored.

Consistent with the effects on biomass production, rhizobial inoculation and phosphorus pelleting improved significantly the total nitrogen uptake and total Δ nitrogen fixation of *S. saman* in the field at eight months after sowing (Table 7). These data are also in agreement with the results obtained previously from the screenhouse experiment whereby rhizobial inoculation enhanced significantly the total nitrogen uptake and total Δ nitrogen fixation of *S. saman*.

SUMMARY AND CONCLUSIONS

Nodulation, biomass production, and nitrogen fixation of *S. saman* with and without rhizobial inoculation were evaluated by using grassland soils under both screenhouse and field conditions. Results from the screenhouse experiment revealed that nodule biomass is a better parameter than nodule number for assessing the effects of rhizobial inoculation. Furthermore, the increase in biomass production with sampling time was exponential suggesting that acacia must be grown for at least 10-12 weeks in the screenhouse before the effects of inoculation treatments could be demonstrated. Inoculation with rhizobia increased significantly the total nitrogen uptake and total Δ nitrogen fixation of acacia.

The data from the field experiment on nodulation and nitrogen fixation were consistent with those observed in screenhouse experiment. In the case of biomass production, rhizobial inoculation and phosphorus pelleting increased significantly the total biomass of *S. saman* by as much as 229 and 277%, respectively. However, upon combining the two, total biomass production was improved remarkably by as much as 673% based on the uninoculated and non-pelleted control. Therefore, in order to optimize biomass production of *S. saman* in grassland areas, it is necessary to provide nitrogen through rhizobial inoculation and phosphorus through pelleting but utilizing mycorrhizal inoculation as an alternative to phosphorus pelleting should also be explored.

ACKNOWLEDGEMENT

The authors are grateful to Mr. Arnel M. Perez, Research Aide and Miss Romana M. Umali, former Science Research Assistant from BIOTECH, for their assistance in the conduct of screenhouse and field experiments. The recommendations and suggestions of Prof. Santiago Alviar of the UPLB Computer Services Unit concerning the statistical aspect of this study are also gratefully acknowledged. Similarly, sincere thanks go to Dr. Shiro Higashi and Ms. Angela dela Cruz for their preparation of the scanning electron micrographs of *Rhizobium* spp. isolates BEs-3 and BEs-4, respectively.

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Table 1. Effect of rhizobial inoculation and sampling time on the total nitrogen uptake and total Δ nitrogen fixation of *S. saman.**

	Total Nitrogen Uptake (mg/pot)	Total Δ Nitrogen Fixed (mg/pot)
Treatment/Isolate		
Uninoculated	310.64 b	0.00 b
BEs-3	340.43 a	38.73 a
Sampling Time (Weeks after sowing)		
2	–	–
4	36.54 f	1.85 a
6	86.83 f	4.72 a
8	152.65 e	3.82 a
10	253.11 d	21.50 a
12	378.32 c	8.68 a
14	522.40 b	33.41 a
16	548.97 b	46.80 a
18	625.47 a	34.15 a

*Any two means within a parameter and within a variable followed by the same letter are not significantly different at 5% level based on DMRT.

Table 2. Effect of rhizobial inoculation and phosphorus pelleting on the nodule number (no/4 plants) of *S. saman* grown under field conditions at 8 months after sowing*.

	Nodule Source		
	Primary Root	Lateral Root	Primary + Lateral
Treatment/Isolate			
Uninoculated	4.57 a	40.00 a	44.57 b
BEs-3	5.88 a	61.75 a	67.62 ab
BEs-4	9.25 a	73.12 a	82.38 a
P-Pelleting			
O-P	5.36 a	61.09 a	66.45 a
P-pelleted	7.83 a	57.25 a	65.08 a

*Any two means within a parameter and within a variable followed by the same letter are not significantly different at 10% level based on DMRT.

Table 3. Effect of rhizobial inoculation and phosphorus pelleting on the nodule dry weight (mg/4 plants) of *S. saman* grown under field conditions at 8 months after sowing*.

	Nodule Source		
	Primary Root	Lateral Root	Primary + Lateral
Treatment/Isolate			
Uninoculated	8.15 b	92.41 b	100.55 b
BEs-3	20.53 b	194.67 ab	215.20 b
BEs-4	72.20 a	317.32 a	389.52 a
P-Pelleting			
O-P	6.88 b	77.70 b	84.58 b
P-pelleted	60.27 a	324.00 a	384.28 a

*Any two means within a parameter and within a variable followed by the same letter are not significantly different at 10% level based on DMRT.

Table 4. Mean interaction effect of rhizobial inoculation and phosphorus pelleting on the nodule dry weight (mg/4 plants) from the lateral and primary + lateral roots of *S. saman* grown under field conditions at 8 months after sowing*.

Treatment/Isolate	Lateral		Primary + lateral	
	O-P	P-pelleted	O-P	P-pelleted
Uninoculated	60.50 c	116.33 c	61.77 c	129.64 c
BEs-3	60.70 c	328.63 b	66.30 c	364.11 b
BEs-4	107.60 c	527.05 a	119.97 c	659.08 a

*Any two means within a parameter followed by the same letter are not significantly different at 10% level based on DMRT.

Table 5. Effect of rhizobial inoculation and phosphorus pelleting on the root, shoot, and total biomass production of *S. saman* grown under field conditions at 8 months after sowing*.

Variable	Root Weight (g/4 plants)	Shoot Weight (g/4 plants)	Total Biomass (g/4 plants)
Treatment/Isolate			
Uninoculated	0.96 b	2.53 b	3.59 b
BEs-3	2.31 a	7.68 a	10.20 a
BEs-4	2.80 a	8.62 a	11.81 a
P-Pelleting			
O-P	0.94 b	2.56 b	3.58 b
P-Pelleted	3.10 a	9.99 a	13.48 a

*Any two means within a parameter and within a variable followed by the same letter are not significantly different at 10% level based on DMRT.

Table 6. Mean interaction effect of rhizobial inoculation and phosphorus pelleting on the root, shoot and total biomass production of *S. saman* grown under field conditions for 8 months*.

Parameter/P-Pelleting	Treatment/Isolate		
	Uninoculated	BEs-3	BEs-4
Root Weight (g/4 plants)			
O-P	0.80 b	0.90 b	1.08 b
P-pelleted	1.08 b	3.72 a	4.52 a
Shoot Weight (g/4 plants)			
O-P	1.62 b	2.58 b	3.25 b
P-pelleted	3.22 b	12.77 a	14.00 a
Total Biomass (g/4 plants)			
O-P	2.48 c	3.54 bc	4.45 b
P-pelleted	4.42 b	16.85 a	19.17 a

*Any two means within a parameter followed by the same letter are not significantly different at 10% level based on DMRT.

Table 7. Effect of rhizobial inoculation and phosphorus pelleting on the total nitrogen uptake and total Δ nitrogen fixation of *S. saman* at 8 months after sowing in the fields*.

Variable	Total Nitrogen Uptake (mg/4 plants)	Total Δ Nitrogen Fixed (mg/4 plants)
Treatment/Isolate		
Uninoculated	88.48 b	0.00 b
BEs-3	205.01 a	136.09 a
BEs-4	241.31 a	166.11 a
P-Pelleting		
O-P	89.77 b	21.74 b
P-pelleted	266.87 a	159.98 a

*Any two means within a parameter and within a variable followed by the same letter are not significantly different at 10% level based on DMRT.

Fig. 1. Transmission electron micrograph of Rhizobium sp. isolate.

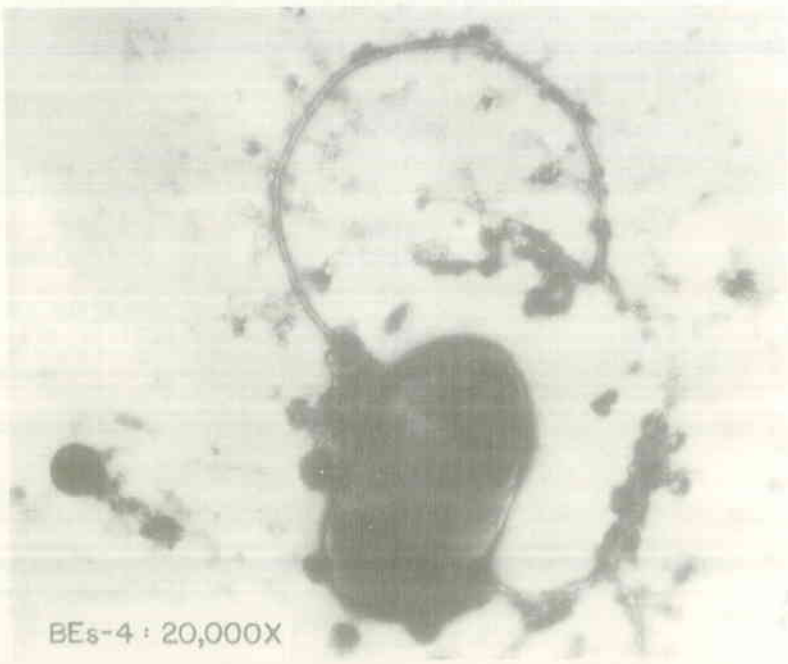
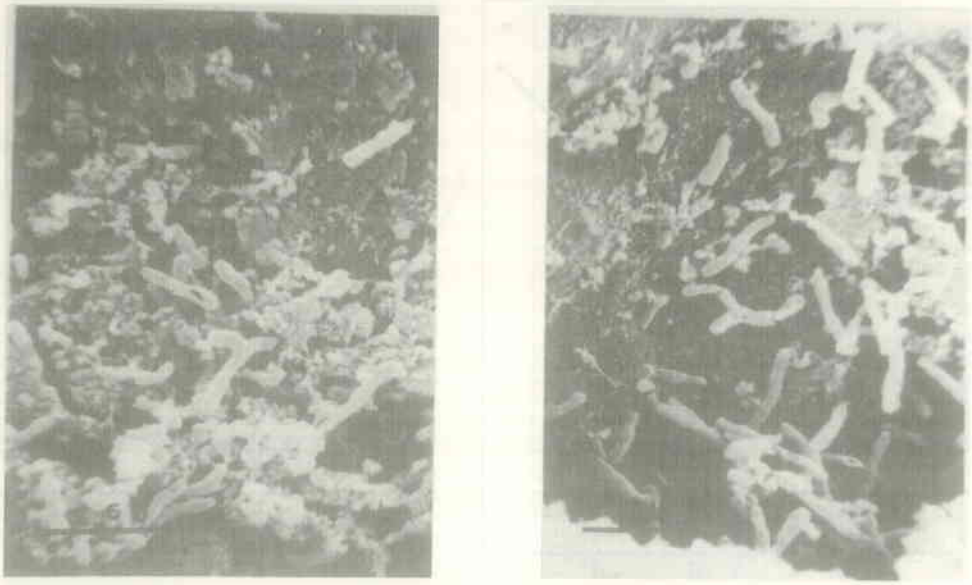
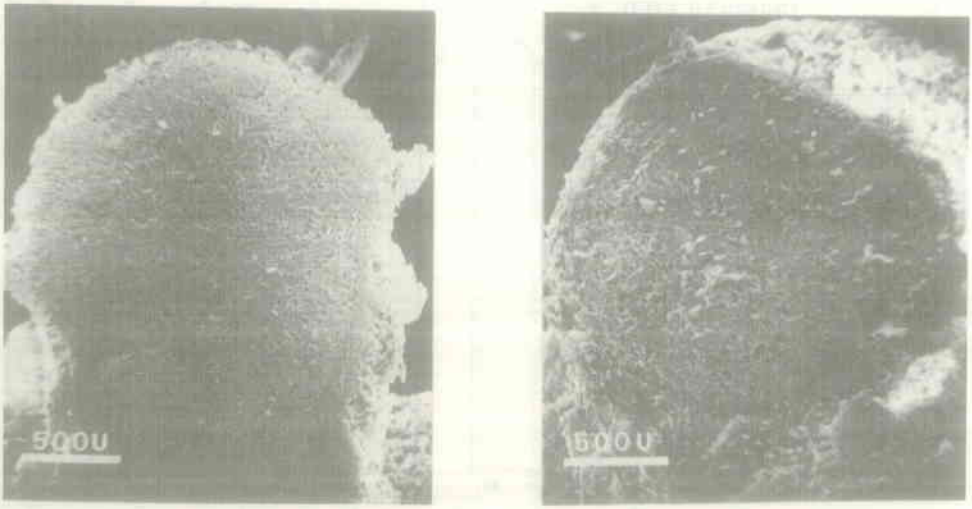


Fig. 2. Transmission electron micrograph of Rhizobium *sp. isolate.*



Samanea saman (Jacq.) Merr.

Fig. 3. Scanning electron micrographs of bacteroids inside the root nodule of S. saman. *(M = 6μ).*



Samanea saman (Jacq.) Merr.

Fig. 4. Scanning electron micrograph of acacia root nodule, longitudinal (left) and cross (right) sections.

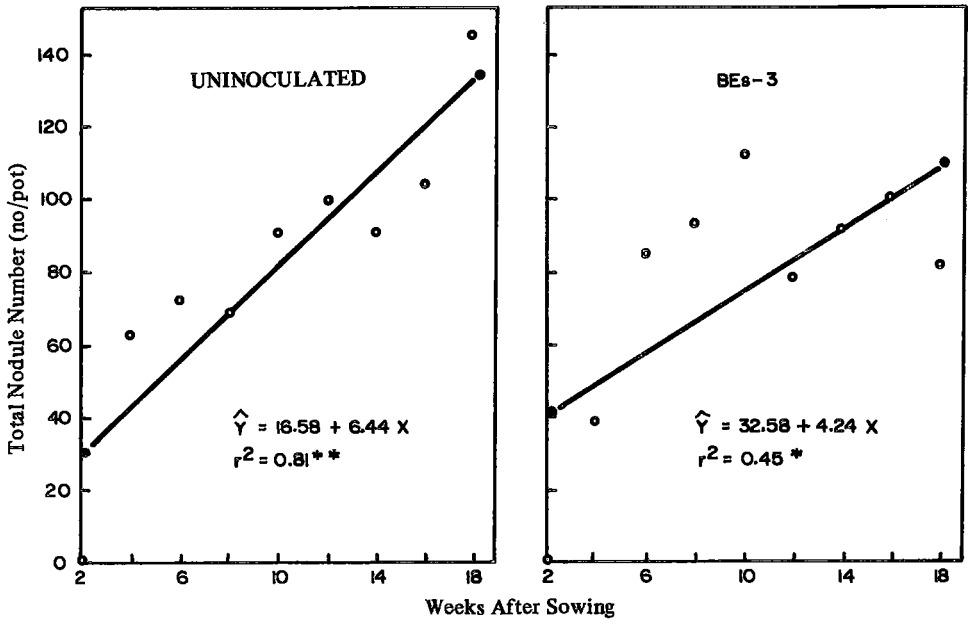


Fig. 5. Regression of the total nodule number with sampling time (weeks after sowing) for S. saman *with and without rhizobial inoculation (○ = observed value and ● = predicted minimum and maximum values).*

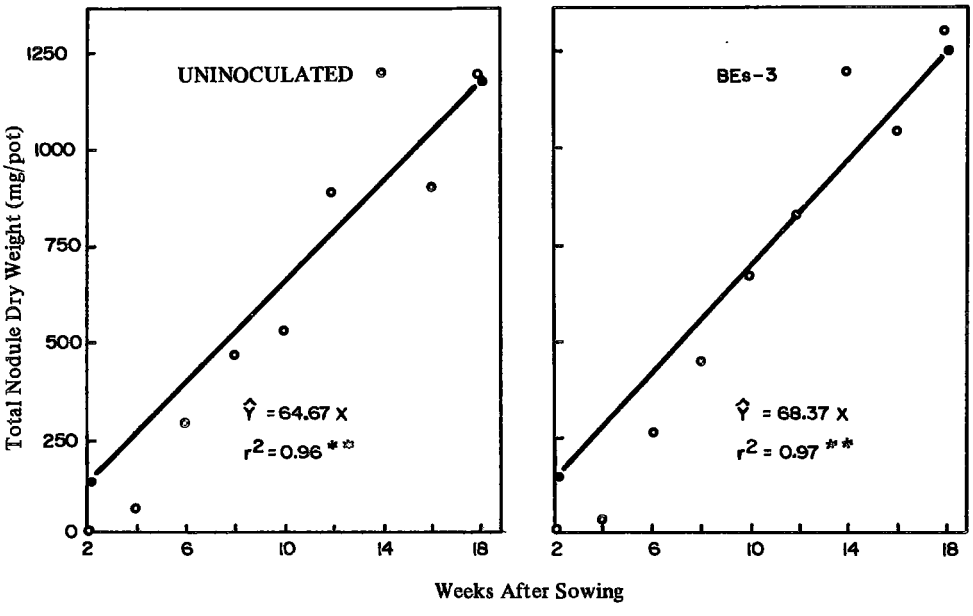


Fig. 6. Regression of the total nodule dry weight with sampling time (weeks after sowing) for S. saman *with and without rhizobial inoculation (○ = observed value and ● = predicted minimum and maximum values).*

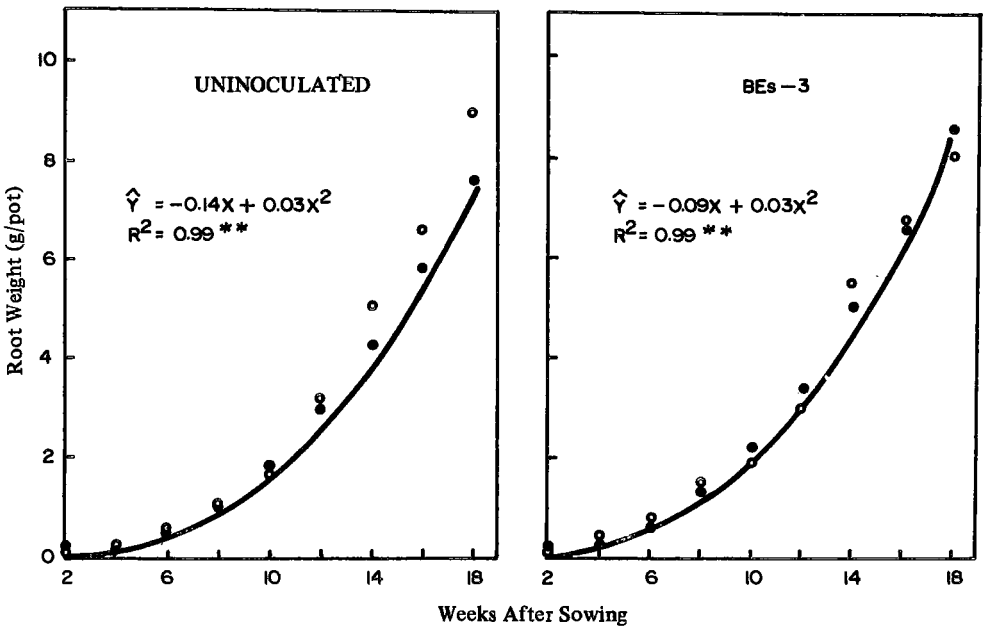


Fig. 7 Changes in the root weight of S. saman *as affected by rhizobial inoculation (○ = observed value and ● = predicted value).*

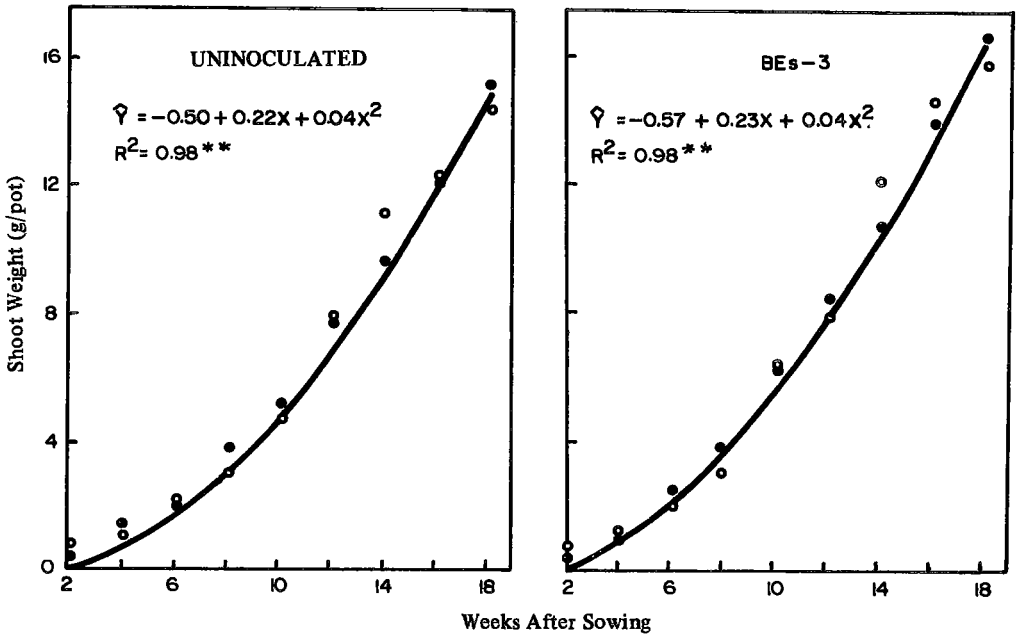


Fig. 8. Changes in the shoot weight of S. saman *as affected by rhizobial inoculation (○ = observed value and ● = predicted value).*

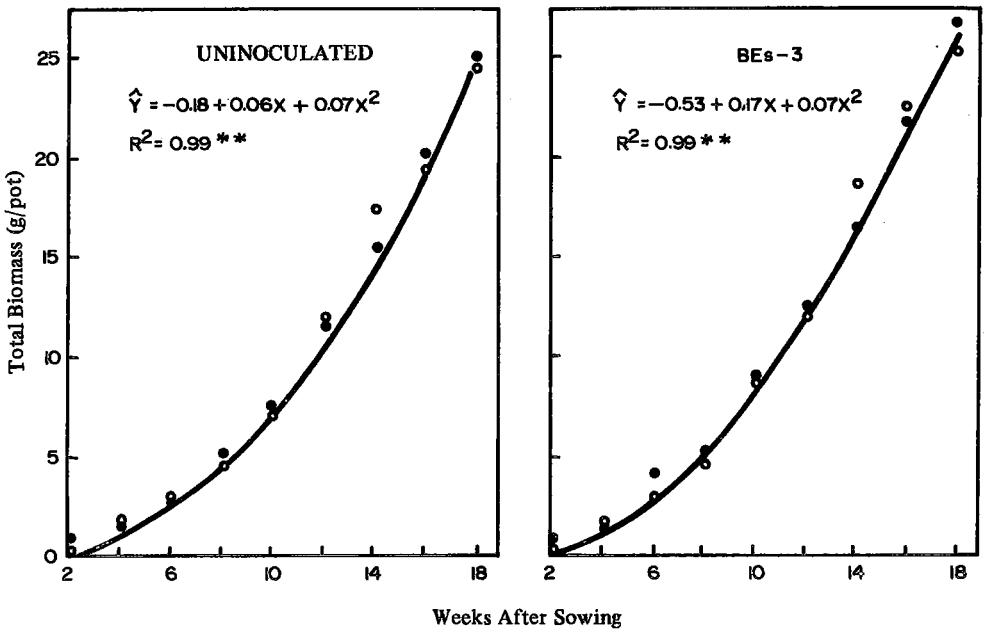


Fig. 9. Changes in the total biomass production of S. saman *as affected by rhizobial inoculation (o = observed value and • = predicted value).*

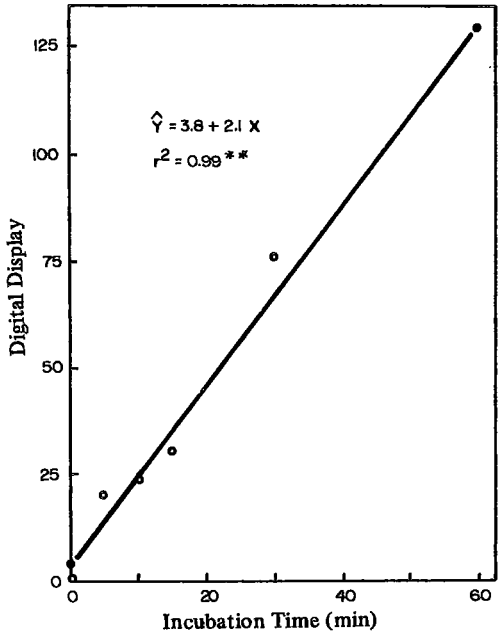


Fig. 10. Regression of digital display (ethylene peak) with incubation time for S. saman *grown under greenhouse conditions (o = observed value and • = predicted minimum and maximum values).*

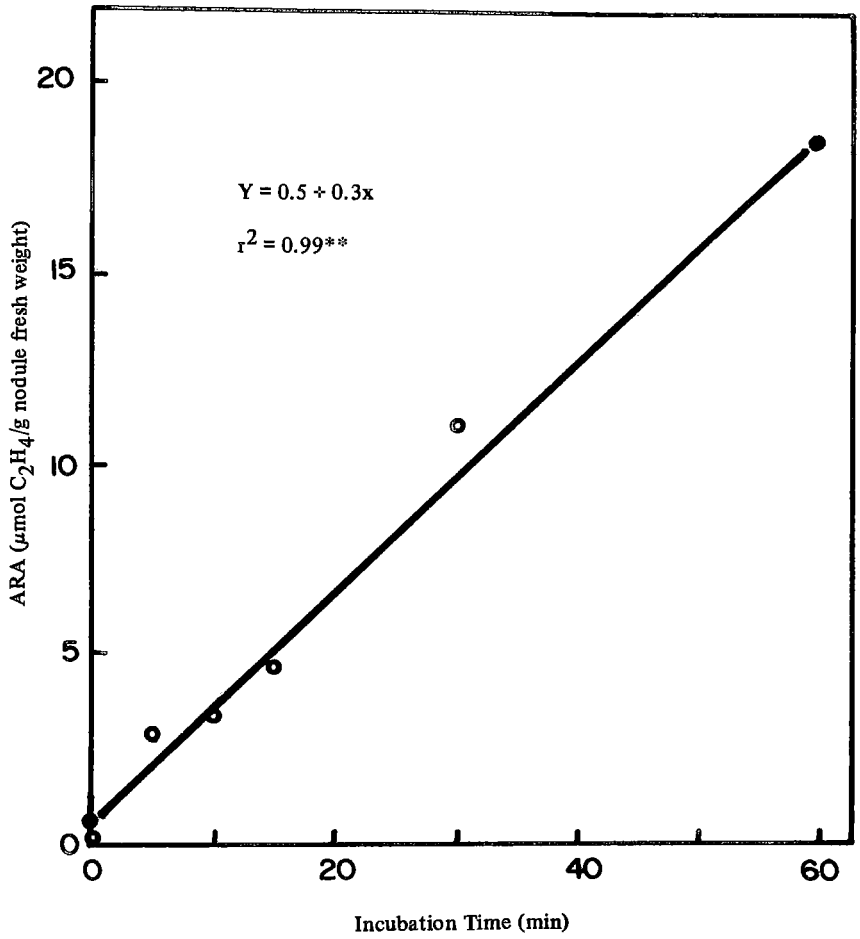


Fig. 11. Time course of acetylene-reducing activity (ARA) of S. saman *grown under greenhouse conditions (○ = observed value and ● = predicted minimum and maximum values).*

ENHANCEMENT OF GROWTH AND NITROGEN FIXATION OF *ALBIZIA PROCERA* (ROXB.) BENTH, ("AKLENG PARANG")*

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ABSTRACT

Rhizobial inoculation enhanced the growth and nitrogen fixation of *Albizia procera* (Roxb.) Benth. ("akleng parang") significantly. Pelleting with either phosphorus or phosphorus plus lime likewise improved growth. However, seed: pelleting material ratio and molybdenum concentration did not have any significant effect on its growth and nitrogen fixation.

INTRODUCTION

Deforestation in the Asia-Pacific region continues at an alarming rate of 5,000 hectares per day (UNEP, 1982) against the background of a seemingly futile forest management and reforestation efforts of government agencies to avert a catastrophic denudation of this region. This results in a deforestation rate of 100,000 to 250,000 hectares per year.

It is not suprising, therefore, that approximately 5 million hectares of denuded or degraded uplands are covered with grass vegetations that are scattered throughout the country today (Llapitan, 1983). To reforest/regenerate these denuded uplands, the Philippine government is spending an average of ₱6 billion annually (Anonymous, 1979). A major fraction of this expenditure goes to fertilization because large amounts of nutrient elements particularly nitrogen and phosphorus are required for the establishment of reforestation/regeneration species in the grassland. Grassland areas are generally deficient in nitrogen or if nitrogen is present in large amounts, it is in the organic form which is not available to the plant. This suggests that nitrogen availability is one of the major factors affecting the productivity of grassland ecosystem (Paul *et al,* 1971).

Another problem in the reforestation/regeneration of grassland areas is the high acidity of the soil or low pH. Generally, this is due to either leaching of basic cations such as K, Ca, and Mg or loss of bases with the surface soil through erosion. Low soil pH

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is associated with low availability of phosphorus and molybdenum which are essential for the enhancement of the effectiveness of the legume-rhizobia symbiosis, an association which could be the key to providing the nitrogen required for the establishment of reforestration/regeneration species in grassland areas.

The choice of a reforestation/regeneration species is critical because of the aforementioned problems. Apparently, leguminous trees are the most suitable for such harsh environments. Some of the leguminous species being recommended by the Bureau of Forest Development (BFD) for reforestation/regeneration of degraded uplands are *Albizia procera,* "akleng parang", *Samanea saman,* acacia, *Pterocarpus indicus,* narra and *Gliricidia sepium,* "kakawati": This paper focuses only on *A. procera.* It is a moderately fast-growing tree which is able to grow on stony, dry, shallow, and acidic soil (NAS, 1979). It bears root nodules; hence, it may have the capacity to provide its nitrogen requirement through fixation of atmospheric nitrogen. It is also drought tolerant. Consequently, it has been identified as a potentially promising species for the regeneration of degraded uplands in almost all regions of the Philippines (PCARRD, 1982).

Very little research work has been done on *A. procera* both locally and internationally (Manguiat, 1982; Quiniones, 1983). Locally, inoculation studies on *A. procera* started about four years ago when its potential for regeneration of marginal uplands was initially recognized (Manguiat and Padilla, 1982). Therefore, if the full potential of *A. procera* as a reforestation/regeneration species is to be exploited, critical factors affecting its growth and nitrogen fixation should be studied. The present investigation attempted to assess the influence of rhizobial inoculation, pelleting material, seed: pelleting material ratio, molybdenum concentration and their interactions on the performance of *A. procera* grown in an acidic grassland soil under screenhouse conditions.

MATERIALS AND METHODS

Bulk surface soil samples (0-15 cm) of Luisiana clay, an acidic soil with a pH of 4.6 and an organic matter content of 1.21%, were collected from a grassland area near the boundary of Cavinti and Luisiana, Laguna. These were air-dried, pulverized to pass through a 1-cm sieve, and th n weighed into clay pots. Each pot was filled with 7 kg of soil and a blanket application of K was done at the rate of 60 kg K_2O/ha by mixing the muriate of potash within the top 5 cm of soil. The soil was moistened with water before the seeds were sown into each pot.

The experimental design was a 2 x 4 x 2 x 2 factorial in a completely randomized design (CRD) with three replications. The different factors were (a) treatment/isolate (uninoculated and BAp-2), (b) pelleting material (none, lime, phosphorus, and phosphorus plus lime), (c) seed:pelleting material ratio (1:0.5 and 1:1.0), and (d) molybdenum concentration (0 and 500 ppm). Seeds of *A. procera* were scarified by soaking them in concentrated sulfuric acid for 10 minutes. The scarified seeds were washed thoroughly with tap water, rinsed several times with distilled water, and then air-dried. One-half of the total number of scarified seeds was inoculated with *Rhizobium* sp. isolate BAp-2 (Fig. 1) which was supplied in the form of soil-based inoculant. The inoculant carrier

was constituted out of plantation forest soil, ipil-ipil charcoal, and wood ash which were sterilized after mixing. The inoculation rate was 10^6 rhizobial cells per seed. A neutral 40% gum arabic solution was used as a sticker to enable the inoculant to stick well onto the seeds. The remainder of the seeds was left uninoculated.

Both the uninoculated and inoculated seeds were pelleted with either lime (+ L), phosphorus (+ P), or phosphorus + lime (+ P + L). One fourth of the total number of seeds was left non-pelleted (– P – L) to serve as control. The pelleting materials were passed through a 60-mesh sieve and either a 1:0.5 or 1:1.0 ratio by weight of seed to pelleting material was used. A sticker (40% gum arabic) was also used to ensure good coating. Lime and phosphorus were supplied in the form of guanzon lime (95% $CaCO_3$) and solophos (18% P_2O_5), respectively.

The treated and untreated seeds were sown into each pot. Two weeks after germination, thinning was done and three plants in each pot were maintained until harvest (18 weeks after sowing). The application of molybdenum was done by foliar spray at 5 and 10 weeks after sowing. At harvest, data on the following growth parameters were gathered: (a) plant height, (b) root length, (c) stem diameter, (d) root diameter, (e) root weight, (f) shoot weight, and (g) total dry matter yield. Data on nodule dry weight, nitrogen uptake, and increase in nitrogen fixation due to rhizobial inoculation (Δ nitrogen fixation) were also collected. The nitrogen uptake was calculated by multiplying the dry matter yield by the percentage of total nitrogen in the tissue. The analysis of plant tissue for total nitrogen was performed using the procedure of Jackson (1958). An estimation of the magnitude of Δ nitrogen fixation was made by subtracting the nitrogen uptake of the uninoculated plants from that of the inoculated plants. This approach assumes that both the uninoculated controls and the inoculated plants are fixing atmospheric nitrogen since the uninoculated plants also formed nodule due to the native rhizobia. The Δ nitrogen fixation, therefore, represents the increase in nitrogen fixation due to rhizobial inoculation.

The data were analyzed statistically and comparison of means was made by using Duncan's Multiple Range Test (DMRT) at 5% significance level. Regression analyses were done to describe mathematically the relationships between (a) Δ nitrogen fixation and nodulation and (b) Δ nitrogen fixation and biomass production.

RESULTS AND DISCUSSION

Growth parameters. Table 1 shows the mean effect of rhizobial inoculation, lime-phosphorus pelleting, seed:pelleting material ratio, and molybdenum application on the height, root length, stem diameter, and root diameter of *A. procera.* Except for root diameter, inoculation with *Rhizobium* sp. isolate BAp-2 did not show any significant effect. However, pelleting with either phosphorus or phosphorus plus lime improved the above-mentioned growth parameters significantly over those of either lime pelleting or no pelleting. In all cases, the increase due to phosphorus alone was higher than that due to phosphorus plus lime indicating that phosphorus pelleting alone was sufficient. Obviously, there was a negative interaction between phosphorus and lime when they

were pelleted together. Had they been applied by mixing with the soil, the effect may have been different because liming acid soils would have made phosphorus more available to the plant. This implies that by pelleting, the cost of establishing *A. procera* seedlings in acidic soils could be reduced significantly since lime would no longer be needed, at least initially.

The data on the interaction effect between rhizobial inoculation and lime-phosphorus pelleting on the stem and root diameters also revealed that whether the seeds were inoculated or not, phosphorus alone was the best pelleting material (Table 2). Lime alone did not show any effect compared with the control (no phosphorus and no lime). However, the combination of phosphorus and lime had a significant effect in the case of the inoculated seeds but the effect was less pronounced than that of phosphorus alone.

Inoculation of *A. procera* seeds with *Rhizobium* sp. isolate BAp-2 enhanced significantly the root, shoot, and total dry matter (biomass) production (Table 3). These findings are in agreement with the results reported earlier (Manguiat and Padilla, 1982). As in the previous data (Table 1), significant increments in root weight, shoot weight, and total dry matter yield were obtained through pelleting with either phosphorus or phosphorus plus lime. On the other hand, seed:pelleting material ratio and molybdenum concentration had no significant effects. Apparently, the soil used had an adequate level of molybdenum inspite of its low pH although acidic soils are normally expected to have low levels of available molybdenum.

The fact that there was no significant difference between the effects of 1:0.5 and 1:1.0 seed:pelleting material ratio is by itself very significant. In a previous paper (Manguiat and Padilla, 1982) it has been reported that by using a 1:1 seed:pelleting material ratio, phosphorus pelleting had a significant beneficial effect on growth and nodulation of *A. procera*. Based on the data from the present study, it would be possible to reduce the amount of phosphorus to be used for pelleting by 50% without sacrificing its effectiveness in biomass production. This means that the cost of pelleting material could be reduced similarly by 50%; thus, it would be more advisable to use a 1:0.5 instead of 1:1.0 seed:pelleting material ratio.

The mean interaction effect of rhizobial inoculation and lime-phosphorus pelleting on the total dry matter yield of *A. procera* indicates that the influence of rhizobial inoculation is enhanced significantly by coupling it with phosphorus pelleting (Table 4). Rhizobial inoculation alone had no effect on total dry matter (biomass) production but phosphorus pelleting alone had a significant effect. However, the effect was much more pronounced when rhizobial inoculation and phosphorus pelleting were combined. These trends were similar to those in the case of stem and root diameters.

Nodulation and Δ nitrogen fixation. Nodulation was assessed based on the nodule dry weights from primary, lateral, and primary + lateral roots (Table 5). Rhizobial inoculation improved significantly the nodule dry weight from the primary but not from the lateral roots. This is expected because the introduced rhizobia are able to colonize the root hairs from the primary roots better than those from the lateral roots in as much as the rhizobia were coated onto the seeds.

The influence of the pelleting material on nodulation was similar to those on biomass production and other growth parameters. On the other hand, the seed:pelleting material ratio had no influence on the total nodule dry weight (primary + lateral roots) of the plant. In the case of the primary roots, however, lower amount of pelleting material favored nodulation implying that the nodulating ability of the introduced rhizobia may have been reduced through direct contact with higher amounts of pelleting materials. In contrast, the nodulation on the lateral roots was substantially enhanced when a greater amount of pelleting material was used.

Among the uninoculated plants, the application of 500 ppm molybdenum did not increase significantly the nodule dry weights from primary roots regardless of the pelleting material (Table 6). Nevertheless, among the inoculated plants, pelleting with either phosphorus or phosphorus plus lime resulted in a significant increase in nodule dry weight upon application of molybdenum.

Table 7 presents the mean influence of the variables studied on nitrogen uptake and Δ nitrogen fixation of *A. procera*. A substantial increase in the total Δ nitrogen fixation but not in the total nitrogen uptake of the plant was obtained through rhizobial inoculation. In the case of phosphorus pelleting, total nitrogen uptake, as well as total Δ nitrogen fixation, was increased remarkably. Neither the seed:pelleting material ratio nor molybdenum concentration affected significantly the total nitrogen uptake and total Δ nitrogen fixation.

Correlation and regression analyses. All growth parameters, with the exception of root length, were highly and positively correlated with the nodule dry weight of either primary, lateral, or primary + lateral roots (Table 8). The data imply that root length is not an appropriate growth character to measure for assessing rhizobial inoculation studies because there is no association between root length and nodulation. However, by knowing the nodulation status in either primary, lateral, or primary + lateral roots, it would be possible to obtain an indication of the expected growth of *A. procera* even in acidic soils. The nodulation status should be helpful therefore in assessing the inoculation needs of *A. procera* to be planted in a particular grassland soil. As in nodule dry weight, total Δ nitrogen fixation was also highly and positively correlated with the different growth parameters, except for root length. In this case, growth parameters may be used therefore as indicators of the magnitude of Δ nitrogen fixation considering that Δ nitrogen fixation is more difficult to measure.

Regression analysis indicates that the relationships between total Δ nitrogen fixation and either shoot weight, root weight, or total dry matter yield were all linear (Fig. 2). Each of the relationships was described by a regression equation; hence, given any Δ nitrogen fixation value within the range of 0-250 mg N/pot, the corresponding root weight, shoot weight, or total dry matter yield (biomass) could be predicted. As illustrated in Fig. 2, it is interesting to note that in the case of *A. procera*, the shoot weight values are very much smaller than the root weight values. Thus, the roots can not be ignored in analyzing plant tissue for nitrogen content if a reasonable estimate of Δ nitrogen fixation is to be obtained.

Fig. 3 shows the regression of total Δ nitrogen fixation with nodule dry weight of either primary, lateral, or primary + lateral roots. In each case, the relationship was described by a linear equation. Within the range of nodule dry weights obtained in this study, total Δ nitrogen fixation could be predicted by knowing the nodule dry weights from either primary, lateral or primary + lateral roots. It appears from these results that if nodulation can be enhanced through rhizobial inoculation and phosphorus pelleting, total Δ nitrogen fixation would increase linearly. A linear increase in total Δ nitrogen fixation could trigger a corresponding enhancement in growth of *A. procera* especially during the seedling establishment stage.

SUMMARY AND CONCLUSIONS

The influence of rhizobial inoculation, pelleting material, seed: pelleting material ratio, molybdenum concentration, and their interactions on growth and nitrogen fixation of *A. procera* grown in an acidic grassland soil were assessed under screenhouse conditions. Inoculation of *A. procera* seeds with *Rhizobium* sp. isolate BAp-2 enhanced growth significantly as indicated by root diameter, root dry weight, shoot dry weight, and total biomass production. Similarly, rhizobial inoculation increased nitrogen fixation significantly. In the case of pelleting material, growth and nitrogen fixation of *A. procera* were enhanced significantly by phosphorus. Pelleting with phosphorus plus lime likewise improved growth but the effect was less pronounced than that of phosphorus pelleting alone. Seed:pelleting material ratio and molydbdenum concentration did not affect growth and nitrogen fixation. However, in almost all parameters considered a 1:0.5 seed: pelleting material ratio was as good as 1:1.0 implying a cost reduction of 50% in terms of pelleting materials.

There was a significant positive interaction between rhizobial inoculation and pelleting material. Stem diameter, root diameter, and total biomass production were improved significantly by combining rhizobial inoculation with either phosphorus or phosphorus plus lime pelleting but the improvement was more pronounced in the case of phosphorus alone. Therefore, in grassland soils, the potential benefits of rhizobial inoculation for *A. procera* could be optimized by combining it with phosphorus pelleting.

All growth parameters, except root length, were highly and positively correlated with either nodulation or Δ nitrogen fixation. Linear relationships were obtained between nodulation and Δ nitrogen fixation. Likewise, the relationships between Δ nitrogen fixation and either root weight, shoot weight, or total biomass production were linear. Regression equations were formulated to predict biomass production based on Δ nitrogen fixation and also to predict Δ nitrogen fixation based on nodulation.

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Table 1. Effect of rhizobial inoculation, lime-phosphorus pelleting, seed:pelleting material ratio, and molybdenum application on the height, root length, stem diameter, and root diameter of *A. procera.**

Variable	Plant Height (cm)	Root Length** (cm)	Stem Diameter (mm)	Root Diameter** (mm)
Treatment/Isolate				
Uninoculated	14.08 a	44.44 a	1.63 a	4.71 b
BAp-2	14.64 a	42.83 a	1.70 a	5.17 a
Pelleting Material				
– P – L	11.85 c	39.74 b	1.52 c	4.34 c
+ L	12.34 c	44.03 ab	1.50 c	4.04 c
+ P	17.66 a	49.60 a	1.95 a	6.25 a
+ P + L	15.60 b	41.17 b	1.68 b	5.14 b
Seed:Pelleting Material Ratio				
1:0.5	14.26 a	43.05 a	1.66 a	4.91 a
1:1.0	14.46 a	44.22 a	1.67 a	4.97 a
Molybdenum Concentration (ppm)				
0	14.40 a	42.85 a	1.67 a	4.97 a
500	14.33 a	44.42 a	1.65 a	4.92 a

*Any two means within a parameter and within a variable followed by the same letter are not significantly different at 5% level based on DMRT.

**Refers to primary root.

Table 2. Mean interaction effect of rhizobial inoculation and lime-phosphorus pelleting on the stem and root diameters of *A. procera.**

Pelleting Material	Stem Diameter (mm)		Root Diameter** (mm)	
	Uninoculated	BAp-2	Uninoculated	BAp-2
– P – L	1.51 de	1.54 de	4.22 cd	4.46 cd
+ L	1.54 de	1.46 e	4.08 d	3.99 d
+ P	1.84 b	2.07 a	5.59 b	6.91 a
+ P + L	1.64 cd	1.72 bc	4.94 bc	5.34 b

*Any two means within a parameter followed by the same letters are not significantly different at 5% level based on DMRT.

**Refers to primary root.

Table 3. Effect of rhizobial inoculation, lime-phosphorus pelleting, seed:pelleting material ratio, and molybdenum application on the mean dry matter production of *A. procera.**

Variable	Root Dry Weight (g/pot)	Shoot Dry Weight (g/pot)	Total Dry Matter Yield (g/pot)
Treatment/Isolate			
Uninoculated	4.87 b	1.34 b	6.30 b
BAp-2	6.45 a	1.60 a	8.15 a
Pelleting Material			
– P – L	3.35 c	0.93 c	4.35 c
+ L	3.08 c	0.86 c	3.99 c
+ P	10.32 a	2.51 a	12.99 a
+ P + L	5.88 b	1.58 b	7.58 b
Seed: Pelleting Material Ratio			
1:0.5	5.33 a	1.42 a	6.85 a
1:1.0	5.98 a	1.52	7.61 a
Molybdenum Concentration (ppm)			
0	5.87 a	1.51 a	7.49 a
500	5.44 a	1.43 a	6.97 a

*Any two means within a parameter and within a variable followed by the same letter are not significantly different at 5% level based on DMRT.

Table 4. Mean interaction effect of rhizobial inoculation and lime-phosphorus pelleting on the total dry matter yield (g/pot) of *A. procera.**

Pelleting Material	Treatment/Isolate	
	Uninoculated	BAp-2
– P – L	3.89 d	4.81 d
+ L	4.36 d	3.63 d
+ P	10.72 b	15.27 a
+ P + L	6.25 cd	8.91 bc

*Any two means followed by the same letter are not significantly different at 5% level based on DMRT.

Table 5. Effect of rhizobial inoculation, lime-phosphorus pelleting, seed:pelleting material ratio, and molybdenum application on the mean nodule dry weight (mg/pot) of *A. procera.**

Variable	Nodule Source		
	Primary Roots	Lateral Roots	Primary + Lateral
Treatment/Isolate			
Uninoculated	43.26 b	49.10 a	92.36 a
BAp-2	59.44 a	54.36 a	113.79 a
Pelleting Material			
– P – L	37.95 b	29.66 b	67.61 c
+ L	28.80 b	28.99 b	57.79 c
+ P	71.55 a	94.16 a	165.71 a
+ P + L	67.10 a	54.09 b	121.18 b
Seed:Pelleting Material Ratio			
1:0.5	59.70 a	41.28 b	100.98 a
1:1.0	42.99 b	62.17 a	105.17 a
Molybdenum Concentration (ppm)			
0	53.11 a	51.74 a	104.86 a
500	49.58 a	51.71 a	101.29 a

*Any two means within a nodule source and within a variable followed by the same letter are not significantly different at 5% level based on DMRT.

Table 6. Mean interaction effect of rhizobial inoculation, lime-phosphorus pelleting, and molybdenum application on the nodule dry weight from primary roots (mg/pot) of *A. procera.**

Treatment/Isolate x Pelleting Material	Molybdenum Concentration (ppm)	
	0	500
Uninoculated		
– P – L	21.95 d	46.55 bcd
+ L	23.74 d	32.82 cd
+ P	39.34 bcd	53.72 bcd
+ P + L	81.31 abc	46.65 bcd
BAp-2		
– P – L	62.95 abcd	20.35 d
+ L	30.86 d	27.77 d
+ P	105.93 a	87.20 ab
+ P + L	58.84 abcd	81.59 abc

*Any two means followed by the same letter(s) are not significantly different at 5% level based on DMRT.

Table 7. Effect of rhizobial inoculation, lime-phosphorus pelleting, seed:pelleting material ratio, and molybdenum application on the total nitrogen uptake and total Δ nitrogen fixation of *A. procera.**

Variable	Total Nitrogen Uptake (mg/pot)	Total Δ Nitrogen Fixed (mg/pot)
Treatment/Isolate		
Uninoculated	114.81 a	0.00 b
BAp-2	139.01 a	48.42 a
Pelleting Material		
– P – L	84.97 bc	14.36 b
+ L	73.44 c	4.67 b
+ P	225.84 a	54.51 a
+ P + L	123.39 b	23.29 ab
Seed:Pelleting Material Ratio		
1:0.5	115.04 a	20.12 a
1:1.0	138.78 a	28.30 a
Molybdenum Concentration (ppm)		
0	131.55 a	30.46 a
500	122.27 a	17.96 a

*Any two means within a parameter and within a variable followed by the same letter are not significantly different at 5% level based on DMRT.

Table 8. Correlation coefficients, r, for different growth parameters and nodule dry weight (mg/pot) or total Δ nitrogen fixation (mg/pot) of *A. procera.**

Growth Parameters	Nodule Source			Total Δ Nitrogen Fixed
	Primary Roots	Lateral Roots	Primary + Lateral	
Plant height (cm)	0.44**	0.52**	0.63**	0.36**
Root length (cm)	– 0.05n.s.	0.20n.s.	0.12n.s.	0.15n.s.
Stem diameter (mm)	0.48**	0.68**	0.76**	0.55**
Root diameter (mm)	0.61**	0.70**	0.86**	0.56**
Root dry weight (g/pot)	0.53**	0.76**	0.86**	0.65**
Shoot dry weight (g/pot)	0.54**	0.70**	0.82**	0.58**
Total dry matter yield (g/pot)	0.54**	0.76**	0.86**	0.65**

*Correlation coefficients, r, lower than or equal to either 0.26 or 0.20 are not significant at either 1% or 5% levels, respectively.

** = highly significant.

n.s. = not significant.

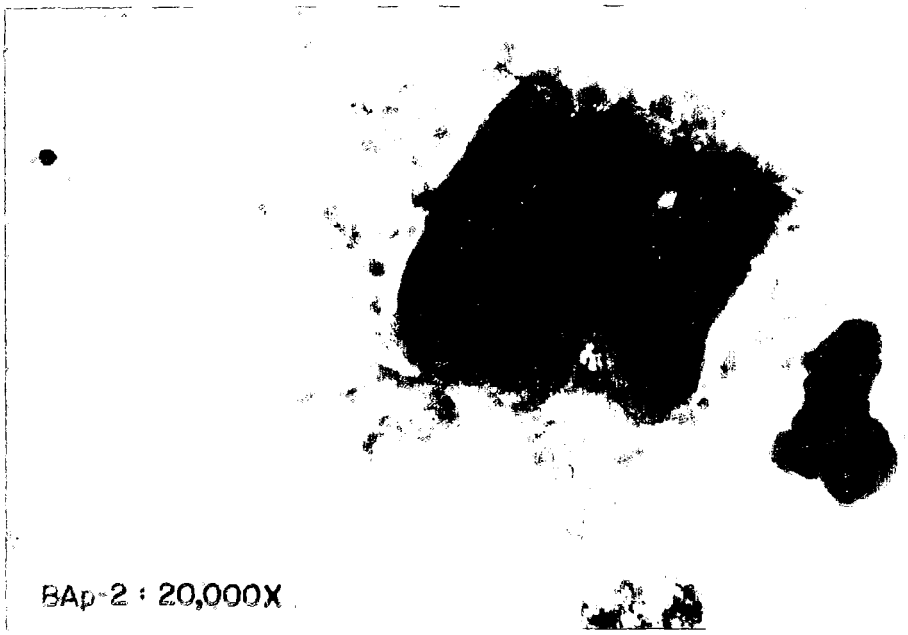

Fig. 1. Transmission electron micrograph of Rhizobium sp. *isolate.*

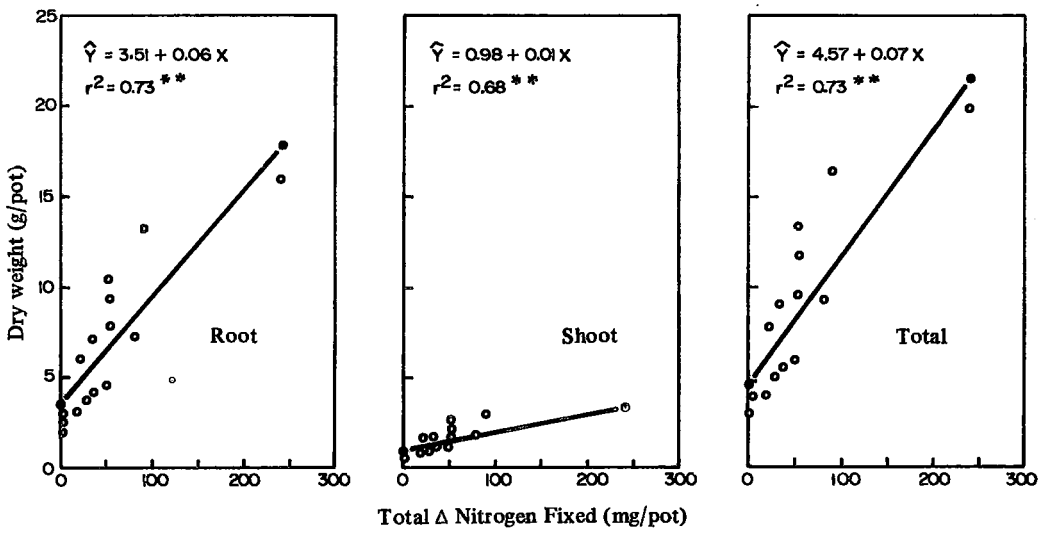

Fig. 2. Regression of the root weight, shoot weight, and total dry matter yield (biomass) with total Δ *nitrogen fixed for inoculated* A. procera *(o = observed value and • = predicted minimum and maximum values).*

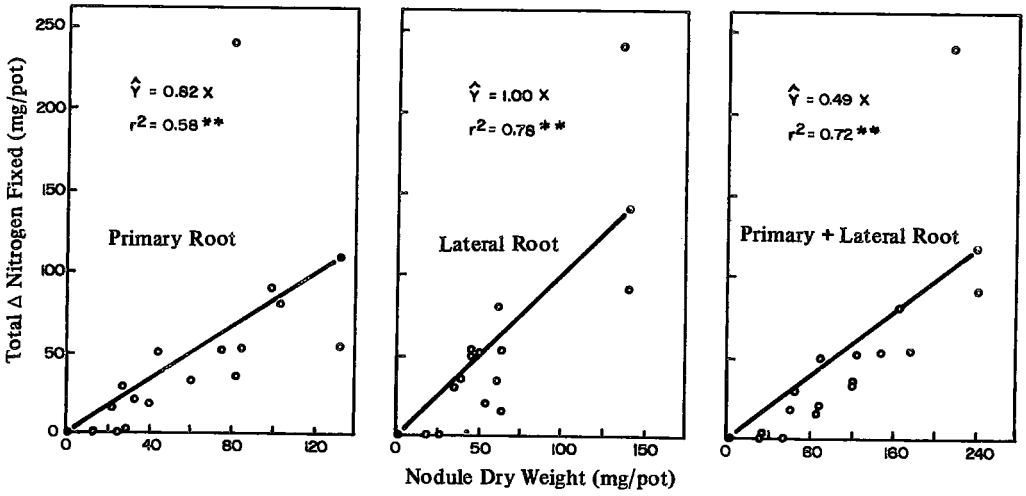

Fig. 3 ***Regression of the total Δ nitrogen fixed with nodule dry weight for inoculated*** A. procera ***(○ = observed value and ● = predicted minimum and maximum values).***

BIOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION OF A PIGMENTED DIAZOTROPH FROM *SACCHARUM SPONTANEUM* L. ROOTS

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ABSTRACT

A nitrogen fixing bacterium isolated from the roots of *Saccharum spontaneum* L. is described. Biochemical and morphological tests conducted on the isolate showed that it is very similar to *Azospirillum lipoferum*. However, the *S. spontaneum* isolate may offer a selective advantage over *A. lipoferum* type strains because of its ability to grow in a wider pH range of 4 to 9.

INTRODUCTION

In the last few years, studies on nitrogen-fixing grass-bacteria associates have accumulated rapidly and the establishment of such associations th ough inoculation studies have been shown to minimize the input of fertilizer nitrogen with increases in yield, nitrogen content and other growth parameters of food crops such as corn (Vlassak, 1982; Hegazi *et al,* 1982 and Jiang *et al,* 1982), wheat (Kapulnik *et a* 1982), barley and sorghum (Subba Rao, 1980) and rice (Lakshman, 1982). The diazotrophic (nitrogen-fixing) bacteria associated with various grass roots have been isolated, characterized and identified using conventional microbiological tests, DNA homology studies and serological techniques (Neyra *et al,* 1977; Tarrand *et al,* 1978). *Azospirillum lipoferum* and *A. brasilense* were reported to be the major soil and root inhabitants responsible for nitrogenase activity in corn and sorghum (Bulow *et al,* 1975; Sloger *et al,* 1976 and Okon *et al,* 1977), wheat (Dobereiner, 1976 and Rennie *et al,* 1982), rice (Barber *et al,* 1976; Watanabe *et al,* 1979; Barraquio *et al,* 1982 and Ladha *et al,* 1982) and in forage grasses (Doboreiner *et al,* 1975).

Diazotrophic bacteria have also been isolated from *Saccharum spontaneum* L. ("talahib"), one of the more dominant wild grasses closely related to sugarcane in the Philippines which can tolerate a wide range of environments and grow abundantly in

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barren soils. A nitrogen-fixing microaerophilic heterotrophic bacterium, TCAL 8, isolated from "talahib" roots, exhibited significant nitrogenase activity by acetylene reduction assay and was shown to enhance the growth of corn plants in field experiments (Garcia *et al,* 1982). Inoculation with this isolate resulted in increases in dry weight and N-uptake of corn and was shown to reduce the input of fertilizer nitrogen from 70 to 30% (Garcia *et al,* 1983). Initial observations on this diazotrophic "talahib" isolate showed that it resembled *Azospirillum* in its pink pigmentation and the formation of white undulating fine pellicles below the surface of semi-solid media. There is a need to characterize this diazotroph both morphologically and biochemically. The purpose of the study therefore, is to compare the biochemical/physiological and morphological characteristics of *S. spontaneum* isolate, TCAL 8, with standard cultures of *Azospirillum* using standard microbiological tests and light and electron microscopic observations of their cellular morphology.

MATERIALS AND METHODS

Source and Maintenance of Diazotrophic Cultures. The pink *S. spontaneum* isolate, TCAL 8, was obtained from the stock culture collection of Dr. M.U. Garcia of the Nitrogen Fixation Group of BIOTECH. For comparison, cultures of *A. lipoferum* sp. USA 5 were obtained from Prof. W. Fernandez, Museum of Natural History, UPLB. All the diazotrophic cultures were maintained in nutrient-agar slants and nitrogen-free semi-solid Dobereiner's medium.

Biochemical and Physiological Characterization of Diazotrophs. The biochemical and physiological tests used to characterize the "talahib" isolate TCAL 8 included combinations of microbiological tests according to the methods of Gibbs and Skinner (1966), Holding and Collee (1971), and Tarrand *et al,* (1978). Tests for carbon metabolism included utilization of different monosaccharides, disaccharides, sugar alcohols, organic acids and starch as sources of carbon and energy, fermentation and gas production, glucose decomposition, starch hydrolysis, while tests for nitrogen metabolism included utilization of dinitrogen, nitrates, ammonia, and glutamate as sources of nitrogen, reduction of nitrates and nitrite and nitrate reduction to gaseous end products, catabolism of certain amino acids such as tryptophan and phenylalanine, urea hydrolysis and thiosulfate reduction to hydrogen sulfide. Other tests determined the ability of the diazotrophs to decompose large biopolymers such as lipids, gelatin, starch and pectin; oxygen requirements of the isolates were determined by growth in nutrient agar-stabs oxidative fermentative activity, catalase activity and degree of microaerophily while reactions to litmus milk was conducted as in standard bacteriological tests. Finally, the ability of the diazotrophs to grow in Dobereiner's medium set at different pHs (2, 4, 6, 7, 9, 11 and 13) and at different temperatures (5°C, 28°C, 38°C, 50°C, 70°C) were also determined.

Morphological Characterization of Diazotrophs. Morphological characterization of diazotrophs included comparisons of colony characteristics in solid nutrient-agar and nitrogen-free medium, and cultural characteristics in agar strokes and in broth cultures. Cellular morphology of diazotrophs was also characterized in terms of shape and size of the cells, Gram-staining reaction of isolates, bacterial motility, presence of bacterial

flagella, bacterial spores, capsules, and intracellular storage granules as observed from light microscopic studies using standard microbiological staining procedures (Conn *et al,* 1960; Gurr, 1962 and Liefson, 1951). Photomicrographs were taken for documentation of the comparative cellular morphological characteristics.

Transmission electron microscopy was also done to further study comparative cell shapes and sizes, cell walls, presence of capsules or slimes, types and wavelengths of bacterial flagella, and presence of intracellular storage granules in the bacterial cells. Prior to electron microscopic observations, preparative techniques involved the aseptic transfer of small amount of growth into small tubes with sterile phosphate buffer at pH 7 and the cell suspension shaken to disperse the cells. A drop of the suspension was pipetted out with fine Pasteur pipettes and dropped onto Collodion-coated or carbon-coated grids, allowed to air-dry for one to two hours, stained with 2% phosphotungstic acid in phosphate buffer for about two minutes. The excess stain was slowly removed with sterile filter paper discs, and the grids were again air-dried for one to two hours inside sterile Petri plates lined with filter paper. When dry, the samples were observed under a transmission electron microscope of the Natural Science Research Center, UP Diliman and electron micrographs were taken for documentation of the cellular morphology of the diazotrophs studied.

RESULTS AND DISCUSSION

Results of biochemical and physiological tests to characterize the pigmented diazotrophic *S. spontaneum* isolate, TCAL 8, and compare with *A. lipoferum,* are summarized in Table 1.

The isolate TCAL 8 and *S. lipoferum* were found to possess the common ability to utilize and ferment the simple monosaccharides glucose, fructose and galactose, the disaccharide sucrose, the pentose arabinose and sugar alcohols, mannitol and sorbitol. Both were capable of glucose fermentation and decomposition (MRVP test) which indicate that TCAL 8 is closer to *A. lipoferum* than to *A. brasilense,* which is non-glucose fermenting (Tarrand *et al,* 1978).

The common inability of TCAL 8 and *A. lipoferum* to utilize the disaccharide maltose may be due to their lack of specific enzymes to breakdown maltose to its glucose units, which can then enter the cell surface and be utilized to provide energy by subsequent oxidation or for growth and protoplasmic synthesis. The isolate TCAL 8, however, differed from *A. lipoferum* in its additional ability to weakly ferment the disaccharide lactose, which was not utilized by the latter. Both were also unable to utilize the polysaccharide starch.

Both diazotrophs utilized organic acids malate, succinate and citrate, as indicated by their common alkaline reaction. Apparently, malate was utilized by both diazotrophs at a faster rate than succinate. Furthermore, the ability of both to grow more abundantly on organic acids than on other sugars verifies earlier reports that growth and nitrogenase activity of diazotrophs, particularly root diazotrophs are supported best by organic acids

(Okon *et al,* 1976; Neyra *et al,* 1977 and Boyle *et al,* 1978) and that although sugar photosynthates are easily translocated to the roots where they enter large carbon pools from which the root diazotrophs derive some of their carbon, dark-CO_2 fixation by the roots utilize the organic acids for their energy requirements (Glatzle *et al,* 1982).

The tests for nitrogen metabolism showed that both diazotrophs were able to grow in nitrogen-free medium, which confirmed their common ability to fix atmospheric nitrogen, but exhibited the most abundant growth in nutrient agar due to the provision of a large supply of growth factors, nutrients and energy sources for rapid multiplication. Enhanced bacterial growth was also observed in the presence of nitrates and ammonium since these could be immediately incorporated into amino acids for protein synthesis and microbial growth. Both diazotrophs were further characterized by the common ability to reduce nitrate and produce gaseous end products from nitrate reduction; both were also able to produce indole from tryptophan catabolism, which may enable them to synthesize plant growth hormones such as indole acetic acid, partially accounting for their observed beneficial effects to the host plant (Tien *et al,* 1979; Gaskins *et al,* 1977 and Hartmann *et al,* 1982). However, the two diazotrophs were incapable of diamination of phenylalanine, urea hydrolysis to ammonia and carbon dioxide, and thiosulfate reduction to hydrogen sulfide.

The "talahib" isolate TCAL 8 and *A. lipoferum* both showed lipolytic activity and weak pectinolytic activity, characteristics which were shown to facilitate infection and colonization of the inner root tissues (Garcia *et al,* 1980); and both were found negative for gelatinase activity. However, TCAL 8 differed from *A. lipoferum* in its additional ability to hydrolyze starch.

Tests for oxygen requirements and oxidative or fermentative activity demonstrated that both diazotrophs were aerobic when grown with fixed-nitrogen sources and microaerophilic when grown in nitrogen-free media, as shown by their growth at or near the surface in stab-inoculated nutrient agar and the formation of a pellicle at a certain depth below the surface in nitrogen-free media. This is in agreement with reports that diazotrophs like *A. lipoferum* required oxygen for growth but fixed atmospheric nitrogen only under microaerophilic conditions (Okon *et al*, 1976; Day *et al*, 1976 and Nelson *et al*, 1978) due to the extreme oxygen lability of the nitrogenase enzyme. TCAL 8 and *A. lipoferum* showed a higher pO_2 requirement due to loss of oxygen-labile nitrogenase complex or more oxygen protection mechanisms since both formed pellicles closer to the surface than other diazotrophic isolates. The degree of microaerophily however, decreased notably both in the presence of nitrates and ammonium, demonstrating a shift from a microaerophilic to a highly aerobic metabolism; this is consistent with reports that *Azospirillum* is highly aerobic when grown with ammonium (Okon *et al,* 1976). Recent studies have further shown that *Azospirillum* could grow at faster rates at high pO_2 in the presence of ammonium, but still actively sought microaerobic conditions by aerotaxis in spite of the presence of the fixed nitrogen (Nur *et al,* 1981 and Okon *et al,* 1980). TCAL 8 was again comparable to *A. lipoferum* since both exhibited mainly oxidative activity and strong catalase activity.

In the litmus milk test, both TCAL 8 and *A. lipoferum* produced an alkaline reaction to litmus milk, followed by soft curdling. Finally, comparison of the growth of the

diazotrophs at different pHs indicated a distinct difference between TCAL 8 and *A. lipoferum* since TCAL 8 was able to grow at a wide acid pH range from 4 to 6 and the alkaline range from 8 to 9, with the optimal pH between 6 to 7; in contrast, *A. lipoferum* was able to grow only at a pH range from 6 to 7. This characteristic could enable the isolate TCAL 8 to tolerate a wide range of environments where most other bacteria will not survive. Growth at different temperatures further showed that both could grow at the temperature range between 28° to 30°C, which classifies them as mesophilic bacteria.

Comparison of the cultural characteristics of TCAL 8 and *A. lipoferum,* is shown in Table 2. Note that both exhibited punctiform to circular, soft, opaque dark pink colonies with smooth-surfaces and entire margins. However, the isolate TCAL 8 was further characterized by an umbonate elevation, a slimy mucoid edge around the colony, a much larger colony size and a darker pink pigmentation than *A. lipoferum* (Figure 1). The pink pigmentation was also noted when grown in agar strokes and in broth culture. Earlier studies have shown that although *Azospirillum* tended to develop a light pigment in nutrient agar medium (Dobereiner *et al,* 1975), certain strains also form very deep pink colonies such as strains Sp13V and SpR68A (Okon *et al,* 1976 and Tarrand *et al,* 1978) and colonies with intense red pigmentation (Eskew *et al,* 1977) which may be spontaneous mutants derived from the ordinary light pink strains. This may also be the case of the isolate TCAL 8. The differences in pigmentation of the pink, dark pink and red strains have been attributed to variations in the cytochrome content of the cells (Eskew *et al,* 1977 and Tarrand *et al,* 1978) or to four different red or yellow carotenoids, which were isolated from membranes of pink and red-pigmented *Azospirillum* strains (Nur *et al,* 1981) grown under aerobic conditions. Carotenoids in pigmented strains were further implicated as an oxygen protection mechanism for nitrogenase (Nur *et al,* 1981) because of their ability to quench singlet oxygen and oxygen radicals (Krinsky, 1979) and act as rigid inserts reinforcing the membrane bilayer, thus reducing oxygen diffusion into the cytoplasm (Nur *et al,* 1982). Likewise, the dark pink pigmentation of the TCAL 8 colonies may also contain carotenoids, which may protect its nitrogenase from inactivation by oxygen, thus accounting for its significant nitrogenase activity and enabling it to grow and fix atmospheric nitrogen closer to the surface of semi-solid media. Aside from the darker pigmentation of the TCAL 8 colonies, the umbonate elevation and formation of a slimy mucoid edge around the colony may also serve as another oxygen protection mechanism by regulating the diffusion of oxygen to the bacterial cells in the colony.

Comparative cellular morphological characterization by light and electron microscopy, also indicated a close similarity between the isolate TCAL 8 and *A. lipoferum* since both were generally characterized as plump, vibroid, curved, or ribbon shaped cells with a slight constriction at the center; both had comparable average cell diameters and cell lengths in nutrient agar medium, although TCAL 8 cells were slightly plumper and shorter than *A. lipoferum* cells, and both were Gram-negative and negative for the presence of bacterial spores. Furthermore, highly refractile darkly staining intracellular granules were distinctly observed at the two terminal ends of most TCAL 8 and *A. lipoferum* cells, which may be considered as a distinguishing characteristic of these diazotrophs. Tarrand *et al,* (1978) similarly describes the large pleomorphic granule-filled forms of *A. lipoferum* as filled with highly refractile granules, probably polyhydroxybutyrate (PHB) granules.

Electron microscopic observations revealed that the cells of isolate TCAL 8 and *A. lipoferum* were commonly characterized by a relatively thick cell envelope enclosing a granulated particulate cytoplasm containing an electron-dense nuclear body or nucleoid. The cells were commonly found embedded in a sticky mucoid substance which could be easily washed away and the presence of a tough electron-dense slime adhering tenaceously to the cell was noted. In some of the cells, a gel-like more defined capsule was observed in both diazotrophs. Other distinguishing cytologic features of TCAL 8 cells, which are shared in common with *A. lipoferum,* were the presence of numerous flagella, some of which were polarly and some laterally inserted into the bacterial protoplast indicating peritrichous flagella (Fig. 2). Both isolates were characterized by large intracellular storage granules, resembling the large membrane-bound PHB granules of other bacterial cells, which were localized at the terminal ends of the cells. This contradicts with reports of large granule-filled forms of *A. lipoferum* (Tarrand *et al,* 1978), prominent lipid inclusions (Eskew *et al,* 1977) or curved cells deformed by fat drops (Rodrigues, 1982), which was actually the basis for its nomenclature (Greek nom *lipus,* fat; Latin verb *fero,* to carry; Mod Latin adj *lipoferus,* fat bearing). This may be explained by reports that at low partial pressures of oxygen, *Azospirillum* formed PHB amounting to about 30% of the cell dry weight; whereas in cells at high pO_2 in the presence of ammonium in the medium, PHB form less than 1% of the cell dry weight (Nur *et al,* 1982). Since the cells were grown in solid nutrient agar medium prior to electron microscopy, the cells observed were probably of the latter type, forming minimal PHB.

In the light of the numerous distinguishing biochemical and morphological characteristics shared by both the TCAL 8 and *A. lipoferum,* it is possible that TCAL 8 may be a variant strain of *A. lipoferum,* which developed a darker pink pigmentation and more mucoid colonies as additional oxygen-protection mechanisms for its oxygen-labile nitrogenase complex. Furthermore, it developed the ability to weakly ferment lactose and partially hydrolyze starch, and may have a selective advantage over *A. lipoferum* type strains because of its ability to grow at a wide acid pH range from 4 to 6 and an alkaline pH range from 8 to 9, where most other bacteria such as *A. lipoferum* will not grow.

The characterization thus presented is however still inadequate for positive identification of this *S. spontaneum* diazotrophic isolate. Further studies should be made to characterize this diazotroph serologically and determine their DNA homology values, which together with the biochemical and morphological characterization, could provide a more positive identification.

SUMMARY

A nitrogen fixing bacterium referred to as TCAL 8 which was isolated from the roots of a native grass, "talahib" (*Saccharum spontaneum* L.) and which was reported to enhance growth and yield of corn is described. The particular bacterium was compared with *Azospirillum lipoferum* biochemically and morphologically.

The isolate TCAL 8 and *A. lipoferum* were shown to possess common ability to utilize and ferment the monosaccharides glucose, fructose and galactose; the disaccharide

sucrose; the pentose, arabinose; and sugar alcohols, mannitol and sorbitol but cannot utilize maltose and starch. These two diazotrophs however, grow better in organic acids such as malate, succinate and citrate.

Both bacteria can reduce nitrate to nitrite and produce indole from tryptophan catabolism. This ability to produce indole compounds may help in understanding the ability of *Azospirillum* to supporting or enhance growth of plants apart from their role in biological nitrogen fixation.

A. lipoferum and TCAL 8 both possess lipolytic and weak pectinolytic abilities. The latter characteristic has been considered to be a factor in easy infection and colonization of inner root tissues of some grasses (Garcia, *et al* 1980).

Both organisms were found to be aerobic in the presence of combined nitrogen (i.e. nitrates and ammonium) but became microaerophilic when grown in nitrogen-free media.

In spite of the various similarities that TCAL 8 shared with *A. lipoferum,* the two differed in the ability to grow in media of varying acidity levels. *A. lipoferum* can only optimally grow in a pH range of 6-7, while TCAL 8 can survive and grow at a wider pH range of 4-9. This tolerance to a wider pH range suggests that TCAL 8 could thrive in environments where most bacteria will not survive.

The two diazotrophs also differ in their colony size, shape and pigmentation. The TCAL 8 colonies are bigger with umbonate elevation, more slimy and darker pink in color. The slime production as well as the dark pigmentation are considered to be part of the oxygen protection mechanism for nitrogenase.

Cytological examination of stained cells revealed that both strains are Gram negative, non-sporulating curved rods, but TCAL 8 cells are more plump than *A. lipoferum* cells.

Electron microscopic examination showed that both have numerous peritrichous flagella and both store numerous poly-hydroxybutyrate (PHB) at low oxygen pressure and form only few PHBs when grown in high oxygen pressure.

Although a good array of common characteristics are shared by the two strains, the darker pink pigmentation developed by TCAL 8 as well as its ability to grow over a wider range of pH tend to indicate that this native isolate may be a variant strain of *A. lipoferum* and that it offers a better selective advantage over *Azospirillum* type strains.

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Table 1. Biochemical and physiological characteristics of *S. spontaneum* isolate, TCAL 8, compared with *A. lipoferum*.

Characteristics	Isolate TCAL 8	*Azospirillum lipoferum*
A. Carbon Metabolism		
1. Utilization[a] and Fermentation[b] of		
Glucose	A +	A +
Fructose	A +	A +
Galactose	A +	A +
Sucrose	A +	A +
Lactose	WA +	S
Maltose	S	S
Arabinose	A +	A +
Sorbitol	WA +	WA +
Mannitol	A +	A +
Malate	K +	K +
Succinate	K +	K +
Citrate	K +	K +
Starch	S	S
2. Methyl Red (MR)-Vogues Proskauer (VP) Test for Glucose Decomposition		
MR Test	+	+
Color of medium	red-orange	red-orange
VP Test	+	+
Color of medium	pink	pink
3. Utilization of Organic Acids		
Citrate Agar	+	+
Amount of growth	moderate	moderate
Malate Broth	+++	+++
Amount of growth	very abundant	very abundant
Succinate Broth	+	+
Amount of growth	moderate	moderate
Succinate Semi-solid	++	++
Amount of growth	abundant	very abundant
B. Nitrogen Metabolism[c]		
1. Utilization of nitrogen test media		
A: + 5 mM nitrate	++	++
B: + 5 mM ammonium chloride	++	++
C: Nutrient Agar	+++	+++
D: Nitrogen-free	++	++
2. Nitrate reduction	++	++
Color with Reagents A and B	bright red brown	bright red brown
3. Nitrite reduction	+	+
Color with Reagents A and B	no change	no change

Table 1 Continued

Characteristics	Isolate TCAL 8	*Azospirillum lipoferum*
4. Nitrate reduction to gaseous end products	+	+
Gas space in Durham tubes	present	present
5. Indole production from tryptophan	+	+
Color on interface	red-brown	red-brown
6. Deamination of phenylalanine	–	–
Color of medium	yellow	yellow
7. Urea hydrolysis	–	–
Color of medium	yellow	yellow
8. Thiosulfate reduction to hydrogen sulfide	–	–
Color of surface	white	white
C. Decomposition of large biopolymers		
1. Lipolysis	+	+
Presence of zone of capacity	present	present
2. Gelatin liquefaction	–	–
State of medium	solid	solid
3. Starch hydrolysis	+	+
Color around inoculation	clear	blue-black
4. Pectin hydrolysis	+	+
Presence of zones of clearing	present	present
D. Oxygen Requirements		
1. Growth in nutrient agar stabs	+	+
Location of growth	at or near surface	at or near surface
Type of organism	aerobic/micro-aerophilic	aerobic/micro-aerophilic
2. Oxidative or fermentative activity		
Acid in open tube only	+	+
Acid in both open and sealed tubes	–	–
Type of metabolism	oxidative	oxidative
3. Catalase activity	++	++
Strength of activity	strong	strong
4. Degree of microaerophily average		
Distance (mm) of pellicle formation in:		
N-free succinate	2.5	2.5
Succinate + nitrate	2.0	2.5
Succinate + ammonium	2.5	2.0
N-free malate	5.0	2.0
Malate + nitrate	2.5	0.0
N-free glucose	6.0	2.0
Glucose + nitrate	2.0	0.0

Table 1 Continued

Characteristics	Isolate TCAL 8	*Azospirillum lipoferum*
E. Reactions to Litmus Milk		
pH of medium	alkaline	alkaline
color	purple	purple
Curd	soft	soft
Casein decomposition	–	–
Reduction of litmus	–	–
F. Growth at different pHs and temperatures		
1. pH variable		
2	–	–
4	+	–
6	++	+
7	+++	++
9	+	–
2. Temperature variable (°C)		
5	–	–
28	++	++
38	+++	+++
50	–	–
70	–	–

Legend:

a: + = growth; S = no growth, no obvious pH change
b: A = acidic (yellow); K = alkaline (blue);
WA = weakly acidic (yellow – green)
c: + = Scant growth; ++ = moderate; +++ = abundant

Table 2. Cultural characteristics and cellular morphological characteristic of TCAL 8 and *A. lipoferum*.

Morphological Characteristics	"Talahib" Isolate TCAL 8	*Azospirillum lipoferum*
A. Cultural Characteristics		
1. In Solid media		
form	punctiform to circular	punctiform to circular
elevation	umbonate	raised
surface	smooth	smooth
margin	entire	entire
density	opaque	opaque
consistency	soft	soft
pigmentation	dark pink	pink

Table 2. Continued

Morphological Characteristics	"Talahib" Isolate TCAL 8	*Azospirillum lipoferum*
diameter at 48 h		
in Nutrient agar	6 mm	4 mm
in N-free medium	0.25 mm	0.3 mm
2. In Agar stroke		
amount of growth	moderate	moderate
consistency	smooth	smooth
form	filiform	filiform
chromogenesis	pink	pink
3. In Broth culture		
surface growth	none	none
sediment (type)	flocculent	flocculent
color of growth	pink	pink
B. Cellular Morphological Characteristics		
1. Cell shape in nitrogen free medium and in nutrient agar	vibrioid, curved or ribbon-shaped	vibrioid, curved or ribbon-shaped
2. Cell sizes (μm)		
average cell dia.	1.2	1.0
average cell lengths in nutrient-agar	4.0	4.09
3. Gram staining reaction	Gram negative	Gram negative
4. Bacterial motility	most very motile	most very motile
manner of movement	some unidirectional some circular and cork-screw motion	some unidirectional some circular and cork-screw motion
5. Presence of		
bacterial spore	negative	negative
slimes	present	present
capsules	present	present
flagella	present	present
type	peritrichous	peritrichous
number	many	many
Storage granules	abundant	scarce

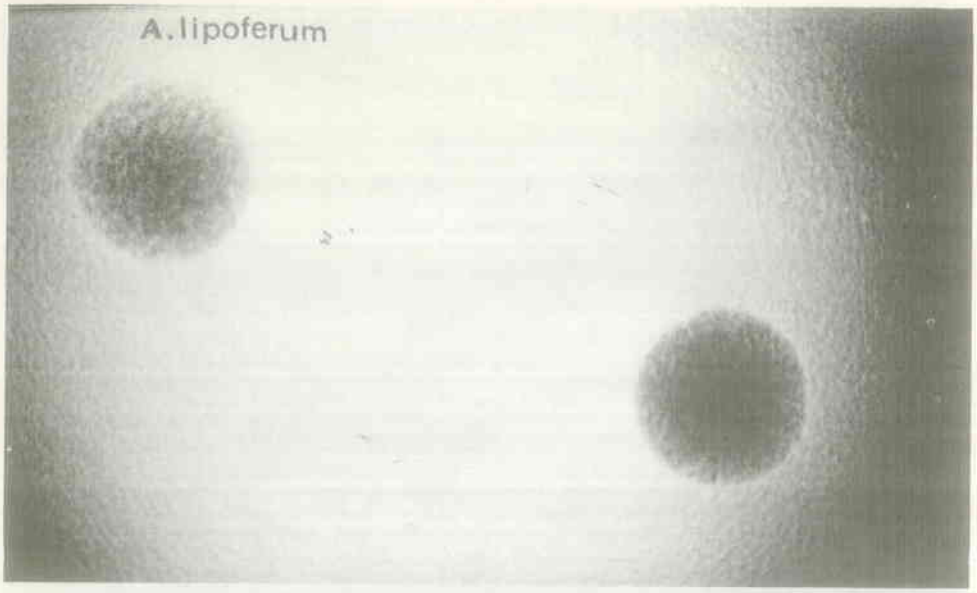

Figure 1a. Photomicrograph of colonies of A. lipoferum *grown in nutrient agar, magnified 25X. Colonies are punctiform to circular, resembling the younger colonies of "talahib" isolate TCAL 8, raised, smooth, soft, have entire margins and opaque but lighter pink pigmentation and smaller size compared with TCAL 8.*

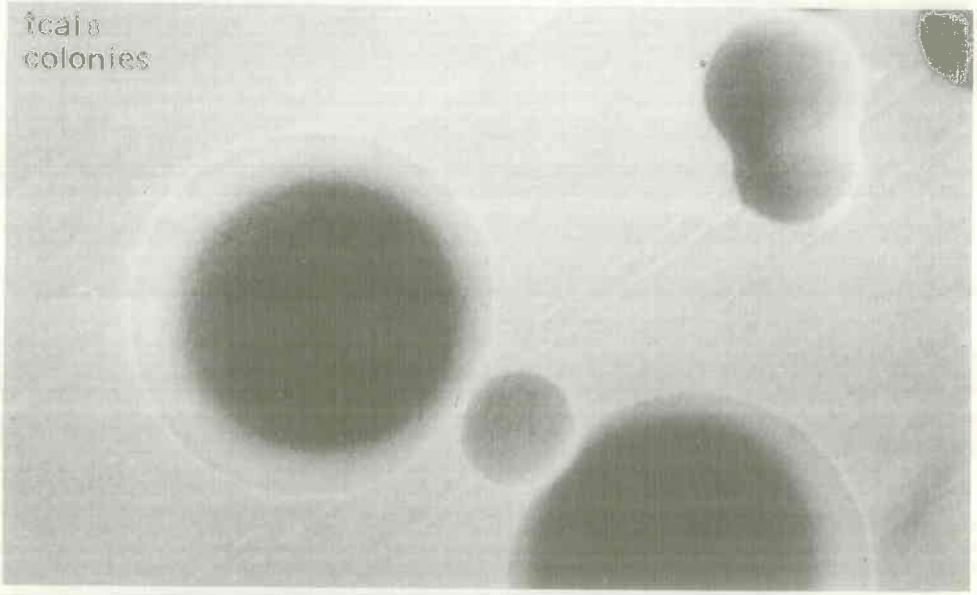

Figure 1b. Photomicrograph of colonies of the "talahib" isolate TCAL 8 in nutrient agar, magnified 25X. Older colonies (larger) are characterized as circular, umbonate, smooth, soft, with an entire margin, opaque dark-pink pigmentation and a mucoid edge around the colony; while younger colonies have lighter pink pigmentation, are smaller in size and have no mucoid edge.

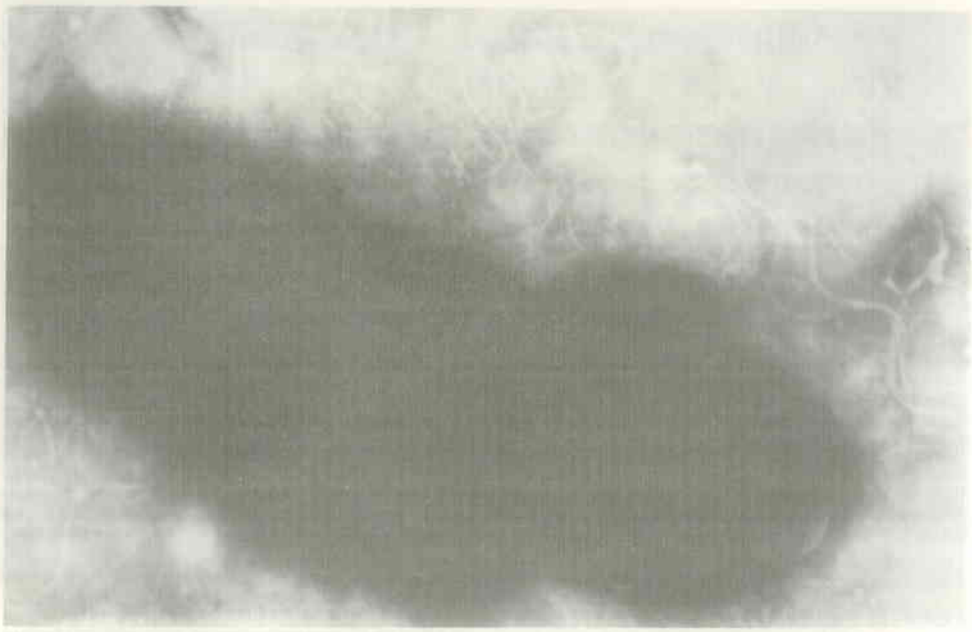

Fig. 2a. Electron micrograph of a vibroid, slightly curved A. lipoferum cell, showing the presence of numerous flagella with numerous coils and waves of small wavelength (M = 20,000 ×).

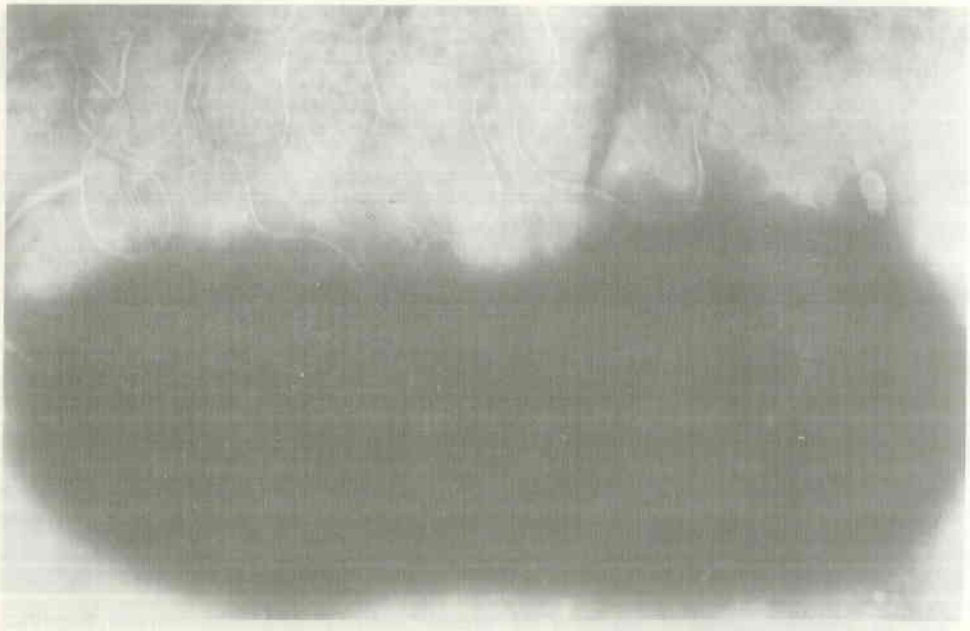

Fig. 2b. Electron micrograph showing vibroid, slightly curved cell of the S. spontaneum [illegible] L. isolate UCAT 8 characterized by numerous polar and lateral flagella (coiled with smaller wavelength than the rice isolate flagella), and the presence of an electron dense slime adhering tenaciously to the cell (M = 20,200 ×).

SEROLOGICAL IDENTIFICATION OF *RHIZOBIUM SPP.* FROM CERTAIN TROPICAL LEGUMES

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ABSTRACT

The technique of agglutination was used for serological identification of *Rhizobium* strains isolated from ipil-ipil (*Leucaena leucocephala*), peanut (*Arachis hypogaea*), mungbean (*Glycine max*) in cell suspension of pure cultures. Antisera of six ipil-ipil strains were tested against twelve different ipil-ipil rhizobial isolates. Six serogroups were identifed using this method although no cross reaction was observed in one of the strains used. Antisera against effective strains of peanut, mungbean and soybean were tested against ineffective strains of each host plant. Agglutination results indicated that the effective strains do not share common antigens with the ineffective strains. The reactions of both heated and unheated culture antigens were the same in most instances but heated culture antigens sometimes exhibited stronger agglutination reactions.

INTRODUCTION

In experiments designed to study the competition between strains of *Rhizobium* applied as inoculum and the naturally occurring population in the soil, a reliable, specific but inexpensive method of identifying the specific strains in the nodules is required.

For accurate identification of the strain of *Rhizobium* forming nodules on leguminous plants, several procedures have been widely used. Most of the studies involved the use of auxotrophy (Johnston and Beringer, 1975) and genetic markers such as antibiotic resistance (Shwinghamer and Dudman, 1973; Josey *et al,* 1979). However, other reports indicate that resistance to some antibiotics may be associated with reduced symbiotic effectiveness (Pain, 1979) decreased infectiveness (Pain, 1979 and Zelasna-Kowalska, 1971) and decreased competitiveness (Bromfield and Jones, 1980).

Serological methods such as the immunofluorescence microscopy (Bohlool and Schmidt, 1970 and Schmidt *et al,* 1968) and the enzyme-linked immunosorbent assay (Kishinevsky and Bar-Joseph, 1978; Berger *et al,* 1979; Kishinevsky and Gurfel, 1980 and Morley and Jones, 1980) have been employed for specific strain identification in legume nodules. A limitation of these methods is the involvement of expensive equip-

ment and resources in carrying out these tests. Less expensive methods such as immunodiffusion and agglutination have also been used for strain identification. Immunodiffusion has been used to indicate occurrence of *Rhizobium* in nodules and in soil (Dudman and Brockwell, 1968; Dudman, 1971 and Kremer and Wagner, 1978). Agglutination has been used to identify nodule bacteria of the *Rhizobium japonicum* and cowpea groups grown in culture (Bushell and Sarles, 1939; Date and Decker, 1965; Johnson and Means, 1965; Koontz and Faber, 1961 and Skrdleta, 1965) or present in the bacterial form in the juice of crushed root nodules (Damirgi *et al,* 1967 and Means *et al,* 1964).

The present paper reports the serological identification of *Rhizobium* isolates from ipil-ipil, soybean, peanut and mungbean by the simple agglutination technique.

MATERIALS AND METHODS

Source and maintenance of cultures. Ipil-ipil *Rhizobium* isolates used in the study are listed in Table 1. Among the twelve ipil-ipil isolates, eight are local or native, one from Australia and three from Hawaii. Rhizobial isolates from soybean, mungbean and peanut used in the study are listed in Table 2. All the strains from these food crops were isolated from local sources. The organisms were maintained on yeast extract mannitol (YEM) agar slants (Fred and Waksman, 1928) which has the following composition: mannitol, 10 g; K_2HPO_4, 0.5 g; $MgSO_4$, 0.2 g; NaCl, 0.1 g; yeast extract, 1.0 g; $CaCO_3$, 0.1 g; Difco agar, 15.0 g; and distilled water, 1.0 L. For immunization of rabbits, they were cultured on YEM broth.

Preparation of antigens. Antigen suspension for immunization were prepared in a way analogous to that for agglutination and immunodiffusion. These were prepared by growing cells on YEM broth for 3-5 days at 25°C under shaken condition. Broth cultures were centrifuged at 2,500 x g for 15 min, supernatant was poured off and then cells were suspended in sterile 0.85% NaCl. Absorbance of cell suspensions was adjusted to a concentration of 10^9 cells per mL using Beckman Spectrophotometer. Population of stock cell suspension was further determined by direct cell count and dilution plating on YEMA. For the analysis of somatic (heat stable) antigens, cells were heated at 80°C (oven) for 30 min. Reactions of heated and unheated cells were compared.

Production of antisera. Antisera against ipil-ipil *Rhizobium* isolates CB81, L5, L57, BL156, tA1600 and tA1602 and against the effective strains of soybean, mungbean and peanut *Rhizobium* were developed by injection of New Zealand white rabbits with native suspensions of heated cells. An immunization protocol of Vincent (1970) was followed in which 10^9 cells per mL were injected into the marginal ear veins of the rabbits according to the following schedule: day 1, 1.0 mL; day 2, 2.0 mL; day 3, 3.0 mL and day 4, 4.0 mL. Sample bleedings were made by cardiac puncture 1 week after the final injection and the sera tested by agglutination. If the test revealed high titer (strong agglutination reaction) the rabbits were test bled again in 5-7 days. The collected blood was allowed to clot at room temperature for 1 h; the clot was cut by ringing the tube and the serum separated after incubating overnight at 4°C. The antisera were further clarified by centrifugation at 860 x g for 10 min. These were stored in the freezer after adding 0.1% merthiolate.

Agglutination. Tube agglutination test was carried out using the procedures of Vincent (1970). Two drops (Pasteur pipet) of the corresponding antiserum was mixed with an equal volume of antigen in an agglutination tube and the mixture was incubated for 4-5 h at 52°C in a water bath.

RESULTS AND DISCUSSION

Agglutination reactions of culture antigens of twelve strains of ipil-ipil *Rhizobium* are recorded in Table 3. As shown in the table, antisera against isolates CB81, L5, L57, BL156, tA1 600 and tA1 602 were used for agglutination test. Cross agglutination tests with heated and unheated antigens of twelve strains indicated that the twelve ipil-ipil isolates belong to six serological groups. The first group consisted of strains L32 and L45, which were related to strain CB81. The second group included strains L6 and L15 which cross-reacted with antiserum to L5. The third group was represented by strain BL 157, which was related to strain L57. Isolate tA1 600, which cross reacted with antiserum to BL156 comprises the fourth group. The fifth group included tA1 601, L32 and L45 which were related to strain tA1 600. The sixth group of tA1 602, did not cross react with any of the antisera used. Strong agglutination reactions were observed with CB81, L5, L57, BL156, tA1 600 and tA1 602 when tested with their homologous antisera. However, when antisera of the mentioned strains were tested against the other isolates, weaker agglutination reactions were observed except L6 where it exhibited maximum reaction when tested against L5 antiserum. It was also observed that among the twelve ipil-ipil isolates L32 and L45 had the widest cross-reactivity in the sense that their antigens cross-reacted to both antisera of CB81 and tA1 600. But the antigenic relationship of CB81 and tA1 600 to L32 and L45 has yet to be ascertained by reacting the antigens of CB81 and tA1 600 to the antisera of L32 and L45. In some instances, heated culture antigens exhibited stronger agglutination reactions than the unheated ones and this could be ascribed to the breakdown of the antigen to a smaller size or from increased release of antigens from the cells during heating (Dudman, 1971 and Berger *et al,* 1979) hence making them more antigenic. The results in Table 3 also show that the place or locality where the organisms originated is not a significant factor to consider when serologically grouping or classifying the isolates because two isolates from the same location are not necessarily antigenically related.

Table 4 shows the agglutination reaction of heated and unheated antigens of *Rhizobium* strains from peanut, mungbean and soybean. Antisera against effective strains from peanut, P3; mungbean, M5 and soybean, S38 were used. Results in Table 5 show that antigens of effective strains, P3, M5 and S38, exhibited strong agglutination reactions with their homologous antisera. On the other hand, no reaction was observed when the antisera were tested with the ineffective strains, P7, M4 and S13. These results indicated that the effective strains do not share common antigens with the ineffective strains hence they are not serologically related and are distinct from each other. No cross-reaction was observed when antisera against mungbean M5 and soybean S38 isolates were tested against peanut and vice versa indicating the non-relatedness of these isolates from different host plants.

The results obtained from this experiment clearly showed that *Rhizobium* isolates could be successfully grouped serologically by the simple agglutination technique using

both heated and unheated culture antigens. It is concluded that, with appropriate standards and controls, the agglutination technique can be used with confidence for the rapid and convenient identification of strains of *Rhizobium* from cultures.

This method may also be used in testing nodule samples such as might be required in studies of *Rhizobium* colonization and nodulation efficiency under different ecological conditions.

SUMMARY AND CONCLUSIONS

Serological identification of *Rhizobium* strains isolated from ipil-ipil, peanut, mungbean and soybean was conducted using the agglutination technique. Six serogroups among the ipil-ipil rhizobial isolates were identified using this method although no cross reaction was exhibited by one of the strains used. Agglutination results, when antisera of effective rhizobial strains of peanut, mungbean and soybean were tested against ineffective strain, indicated that the effective strains do not share common antigen with the ineffective strains. The reactions of both heated and unheated culture antigens were identical for almost all the strains investigated.

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Table 1. Sources of ipil-ipil *Rhizobium* isolates.[a]

Code Name	Source
CB 81	Australia
L5	College Experimental Station, UPLB
L6	College Experimental Station, UPLB
L15	Antipolo, Rizal
L32	Luisiana, Laguna
L45	Alitagtag, Batangas
L57	Luisiana, Laguna
BL156	Novaliches, Metro Manila
BL157	Balukan, Sariaya, Quezon
tA1 600	Niftal, Hawaii
tA1 601	Niftal, Hawaii
tA1 602	Niftal, Hawaii

[a]Isolates were provided by Dr.S.N. Tilo and associates of the UPLB Soil Science Department and BIOTECH, College, Laguna.

Table 2. Sources of *Rhizobium* isolates[a] from some food crop legumes.

Species and Strain No.	Sources
Peanut *Rhizobium*	
*P3	College, Experimental Station, UPLB
P7	San Jose, Batangas
Mungbean *Rhizobium*	
M4	College Experimental Station, UPLB
*M5	College Experimental Station, UPLB
Soybean *Rhizobium*	
S13	Unknown local source
*S38	Unknown local source

[a]Isolates were provided by Dr. E.S. Paterno and associates of the UPLB Soil Science Department and BIOTECH, College, Laguna.

*Effective strains.

Table 3. Agglutination (cross reaction) analysis of heated and unheated antigens of *Rhizobium spp.* isolated from ipil-ipil.

	Agglutination reaction produced with indicated antiserum and form of antigen*											
Antigen	CB81		L5		L57		BL156		tA1 600		tA1 602	
	H^{+}	U^{++}	H	U	H	U	H	U	H	U	H	U
CB81	4+	4+	–	–	–	–	–	–	–	–	–	–
L5	–	–	4+	4+	–	–	–	–	–	–	–	–
L6	–	–	4+	4+	–	–	–	–	–	–	–	–
L15	–	–	2+	2+	–	–	–	–	–	–	–	–
L32	1+	1+	–	–	–	–	–	–	2+	2	–	–
L45	2+	–	–	–	–	–	–	–	2+	1+	–	–
L57	–	–	–	–	4+	3+	–	–	–	–	–	–
BL156	–	–	–	–	–	–	4+	4+	–	-	–	–
BL157	–	–	–	–	2+	1+	–	–	–	–	–	–
tA1 600	–	–	–	–	–	–	1+	–	4+	4+	–	–
tA1 601	–	–	–	–	–	–	–	–	2+	2	–	–
tA1 602	–	–	–	–	–	–	–	–	–	–	4+	4+

*A dash (–) indicates that no agglutination was observed. 4+ to 1+ indicates that maximum or complete to minimum agglutination, respectively, was detected. Underscored indicates a homologous reaction. Antisera used was undiluted and with a titer of 1:512.

+H = heated antigen.

++U = unheated antigen.

Table 4. Agglutination reaction of *Rhizobium* strains isolated from some crop legumes.

Isolate/Antigen	Agglutination reaction produced with antisera of effective strains and form of antigen*					
	P3		M5		S38	
	H^{+}	U^{++}	H	U	H	U
Peanut *Rhizobium*						
P3	4+	4+	–	–	–	–
P7	–	–	–	–	–	–
Mungbean *Rhizobium*						
M4	–	–	–	–	–	–
M5	–	–	4+	4+	–	–
Soybean *Rhizobium*						
S13	–	–	–	–	–	–
S38	–	–	–	–	4+	4+

*A dash (–) indicates that no agglutination was observed. 4+ indicates maximum agglutination. Underscored indicates a homologous reaction.

+H = heated antigen.

++U = unheated antigen.

A COLORIMETRIC PROCEDURE FOR DETERMINING THE NUMBERS OF RHIZOBIA IN LEGUME INOCULA

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ABSTRACT

A colorimetric procedure was developed for determining the numbers of *Rhizobium japonicum* (soybean rhizobia) in legume inocula. The procedure is similar in principle with enzyme-linked immunosorbent assay (ELISA) which employs alkaline phosphatase, anti-rhizobia IgG complex. Some technical parameters that were defined and tested were purification of rabbit-derived antibodies against *Rhizobium japonicum;* conjugation of purified antibodies with an alkaline phosphatase enzyme; heating of bacterial suspensions to destroy non-specific antigens; labelling bacteria with the antibody-enzyme complex; removing non-adsorbed enzyme prior to chemical analysis and defining conditions of the enzymatic assay used to determine cell numbers. The procedure can be used to detect from 10^1 to 10^9 soybean rhizobia/mL but more variable results were obtained at concentrations of 10^1 to 10^5 rhizobia/mL.

INTRODUCTION

Leguminous plants can obtain nitrogen via symbiotic dinitrogen fixation. This is the conversion of atmospheric dinitrogen (N_2) to NH_3 and is performed by *Rhizobium* in nodules of leguminous plants. To ensure an efficient fixation, inocula are often applied to legume seeds at planting. A good inoculant should contain high numbers of rhizobia, both when manufactured and used by farmers. These rhizobia should survive and multiply in the soil. They should also colonize the rhizosphere of the host plant, be infective and fix nitrogen for the plant in an efficient manner. Inocula should contain at least 10^8 viable rhizobia per mL when applied to the seed at the time of planting (Vincent, 1965).

Several methods have been developed and used to determine inoculant quality. The methods used are plate counts (Vincent, 1970); plant grow-out tests (Burton, 1967;

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Hiltbold *et al,* 1980; Skipper *et al,* 1980), Most-Probable-Number (MPN) analyses (Alexander, 1965; Weaver and Frederick, 1972) and fluorescent-antibody microscopy (Schmidt *et al,* 1968; Bohlool and Schmidt, 1970; Jones and Russel, 1972; Schmidt, 1974). Although these methods are available, most are labor intensive and expensive, while some are non-quantitative and require growth chamber and greenhouse facilities.

An improved method is needed for determining the number of infective rhizobia in legume inocula. The method must be rapid, valid, precise, inexpensive and specific (that is, it should work with defined strains of *Rhizobium*). The objective of this study is to develop a colorimetric procedure employing an enzyme-antibody complex to determine the number of *Rhizobium* in legume inocula specifically in pure culture.

MATERIALS AND METHODS

Organisms. The bacterium used in the early phase of the study was strain 80 of *Rhizobium japonicum* (Beltsville serogroup 110) provided by Dr. H.L. Peterson. Strains of *R. japonicum* were also isolated (Vincent, 1965) from a commercial inoculant (Nitragin peat-base soybean inoculant). The bacterial isolates were maintained on yeast extract mannitol (YEM) agar slants.

Characterization and grouping of isolates. The isolates were characterized morphologically and classified according to serology. One isolate was injected into a rabbit either via intravenous injection or intramuscular injection. The antiserum produced against the isolate was used in the agglutination tests (Vincent, 1970) using the test antigen and the other isolates obtained from the inoculant. Antisera were produced against each group of isolates and consequently used to determine the number of rhizobia in legume inocula.

Antisera production. A stock cell suspension was prepared from a fresh, 5-day old broth culture of *Rhizobium japonicum* (approximately 10^9 cells/mL). The cells were harvested by centrifugation at 2,800 x *g* for 60 min at 5°C. The cell pellet was resuspended in 10 mL sterile 0.85% sodium chloride solution. A suspension of approximately 10^8/mL was obtained by both direct microscopic cell counts using a Petroff-Hauser counting chamber and plate counts on YEM agar and heated at 80°C for 30 min. The cells were injected into the ear vein of a rabbit every other day in four increasing doses of 1.0, 2.0, 3.0 and 4.0 mL. The presence of specific antibodies in the serum was checked 7 days after the last immunization. Booster injections were done whenever necessary. Blood was collected 5 days after the booster shot. The serum was separated from the clot after incubation at room temperature for 2 h followed by refrigeration overnight and centrifugation at 2,800 x *g* for 30 min at 5°C. The titer of the serum was 1:1024 as determined by agglutination test (Vincent, 1970) in microtiter plates. Serum was dispensed in vials and stored in the freezer.

Collection and purification of IgG from the rabbit antiserum. Two mL of antiserum were combined slowly (drop by drop) with an equal volume of sterile 36% sodium sulfate solution. After the last drop, the solution was stirred with a magnetic stirrer to com-

plete the precipitation of the IgG. At this point, the IgG was visible as a pinkish white, heavy precipitate. The IgG was removed by centrifugation at 1,100 x *g* at room temperature. The supernatant was decanted and the precipitate was washed by resuspension with approximately 2.0 mL of sterile 18% sodium sulfate solution and centrifugation as above. The final white precipitate was resuspended in 1.0 mL of phosphate buffered saline (PBS), pH 7.4. The IgG was further purified by ultra-filtration using an Amicon Model 52 (UM 10) ultra-filter and then extensively washed with PBS. The IgG concentration was determined using the Lowry's method (Lowry *et al,* 1951).

Preparation of rhizobial antigen for enzyme-linked immunosorbent assay (ELISA). A fresh, broth culture (5-day old and in late exponential growth) was harvested by centrifugation at 2,800 x *g* for 120 min at 5°C using a refrigerated centrifuge. The supernatant was decanted and the cell pellet was washed by resuspension in 0.05 M Tris-HCl saline buffer, pH 8.0 (TBS) followed by centrifugation as above.The cell pellet was resuspended in 100 mL TBS to obtain an approximate concentration of 10^9 cells. This served as the stock cell suspension.

Conjugation of IgG with alkaline phosphatase. To determine the optimum concentration of enzyme-IgG conjugate, varying concentrations of alkaline phosphatase (0.1, 1.0 and 2.0 mg) were added to 1.4 mg IgG and the mixture was allowed to react for 4 h at room temperature, and purified by ultra-filtration as above using 50 mL PBS. The conjugate was removed from the filter by washing it with approximately 2.0 mL PBS. To this 2.0 mL sample, 16 mL of 25% glutaraldehyde were added and the mixture incubated for 30 min to 2 h. The sample was then ultrafiltered with 100 mL TBS after which 2.0 mL TBS containing 1% bovine serum albumin and 0.02% sodium azide was added to the sample. The stock conjugates were stored in brown vials in the freezer.

Determination of number of R. japonicum *with conjugated phosphatase IgG test tube assay.* A procedure similar to the ELISA procedure of Engvall and Perlmann (1971; 1972) with modifications of Voller *et al,* (1976) was employed to determine the numbers of *R. japonicum* in pure culture. The stock cell suspension (see above) was serially diluted in 10-fold steps to provide the following cell concentrations: 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0/mL. Six tubes containing 1 mL of cell suspension were used for each dilution or cell concentration. The cells were heated at 80°C (oven) for 30 min. After heating, 1.0 mL of the conjugate was added to each of the three tubes of all the cell concentrations prepared while the other three tubes served as controls. The samples were incubated at room temperature for 3 h. After labelling, each sample was filtered in a vacuum and washed with 50.0 mL TBS. Metricel filter membranes were employed with 0.2, 0.3 and 0.45 μm pore size. The filter membrane was removed from the funnel immediately after filtration and placed in a flask containing 10.0 mL 10% diethanolamine buffer, pH 9.8 to minimize enzyme denaturation. Five mL of the 0.05 M *p*-nitrophenylphosphate substrate was added to each flask and samples were incubated in a water bath shaker (37°C). The phosphatase reaction was terminated by adding 5.0 mL of 1.0 N NaOH to each flask. Samples were transferred to volumetric flasks (leaving filter in the flasks) and the final volume was brought to 25.0 mL with diethanolamine buffer. Percent transmittance of *p*-nitrophenol in the samples was read at 440 nm against the blank com-

posed of the diethanolamine buffer and 1.0 N NaOH, using a Perkin-Elmer 552 spectrophotometer. Dilutions were prepared so that percent transmittance was between 10.0 and 100.0. Para-nitrophenol produced (mg/L) was obtained using the standard *p*-nitrophenol curve in Fig. 1.

RESULTS AND DISCUSSION

Liquid suspensions of *R. japonicum* (strain 80, serogroup 110) containing from 10^0 to 10^9 cells/mL were used to investigate the sensitivity of ELISA for determining the number of rhizobia in pure culture. Results of earlier experiments where 0.1 or 1.0 mg alkaline phosphatase/mL and 1.4 mg IgG/mL were used in the preparation of conjugate and then coupled by glutaraldehyde for 30 min and incubated with the substrate for 30 min or 2 h, indicated that the limit of detection with the ELISA procedure was about 10^7 rhizobia/mL. At and below 10^6 rhizobia/mL variable and erratic results were obtained. The results suggest that probably 0.1 mg enzyme/mL and 30 min to 2 h incubation time with the substrate were insufficient for an optimum reaction to occur. However, prolonging the incubation time of the samples with the substrate for 4 h increased the sensitivity of the method and enabled the detection of rhizobia in samples containing as few as 3.4×10^4 cells (Fig. 2). Consequently, 4 h incubation period of samples with the substrate was used in subsequent analysis. Clark and Adams (1977) found that extinction values are affected both by the concentration of the conjugate and by the length of the incubation time over a period of 1.0 h when used to detect plant viruses.

Another series of experiments were conducted to determine the effect of higher enzyme concentration on the efficiency and sensitivity of the procedure. Increasing enzyme concentration to 2.0 mg/mL increased the total enzyme activity and the sensitivity of the procedure (Fig. 3). Rhizobial antigens were detected in cell suspensions containing as few as 10^2 cells/mL. Statistical analysis of the data gave a very high correlation (r = 99%) between the log number of cells and the amount of *p*-nitrophenol released during enzyme hydrolysis of the substate.

A similar experiment was conducted as above but the alkaline phosphatase and IgG were coupled by glutaraldehyde for 2 h instead of 30 min. Results reveal that ELISA enabled the detection of rhizobial antigens from 10^9 to as low as 2.5×10^0 cells/mL (Fig. 4). Regression analysis of the data gave a very good correlation (R = 91% to 97%) between the log number of cells and the enzyme activity. Avrameas (1969) reported that glutaraldehyde is the most effective and suitable reagent for producing enzyme protein complexes which retained a part of their enzymatic and immunological specificity.

Additional experiments were conducted using lower cell concentrations (ranging from 10^0 to 10^5 cells/mL) in an attempt to improve the precision of the procedure. Reproducible results were obtained with samples containing as few as 10^3 to 10^4 rhizobia. Below this range, erratic data were sometimes observed probably because of the nonspecific absorption of protein by the Millipore filter membranes. The absorbance values of filter membranes plus conjugate plus substrate (without cells) were almost identical with those containing cells (data not shown).

In a subsequent experiment, it was also shown that there was a very good correlation between the log number of cells and the amount of *p*-nitrophenol released during hydrolysis of the substrate by the enzyme (Fig. 5). This indicates that *Rhizobium* could be detected in a suspension containing as few as 10^0 cells/mL, although it is evident that the assay is most precise with 10^4 to 10^9 rhizobia/mL. These results agree with those reported by Morley and Jones (1980).

SUMMARY AND CONCLUSIONS

A colorimetric procedure was developed for determining the number of *Rhizobium japonicum,* the principle of which is similar to the enzyme-linked immunosorbent assay which employs an alkaline phosphatase, anti-rhizobia IgG complex.

The enzyme-labelled antibody was prepared by mixing the enzyme alkaline phosphatase and IgG, followed by incubation for 4 h at room temperature and purification by ultra-filtration. Conjugation of the enzyme and IgG was promoted by exposure to glutaraldehyde for 2 h at room temperature. The conjugate was purified by ultra-filtration and stored in brown vials in the freezer.

The colorimetric procedure developed in this study involved: (1) precipitation of the rabbit-derived antibody by sodium sulfate and its purification by centrifugation and ultra-filtration; (2) conjugation of 1.4 mg IgG protein with alkaline phosphatase using glutaraldehyde for 2 h at room temperature; (3) heating the cells at 80°C for 30 min; (4) labelling the bacteria with the antibody-enzyme complex at room temperature for 3 h; (5) washing cells to remove non-adsorbed enzyme using millipore filtration; (6) incubating the bacteria with *p*-nitrophenyl phosphate substrate at 37°C for 4 h; (7) addition of 1N NaOH to stop enzyme-substrate reaction; (8) bringing the sample volume to 25 mL with a buffer; and (9) determination of the amount of *p*-nitrophenol formed by using a spectrophotometer.

The developed procedure can detect from as many as one billion to as few as ten rhizobia per mL using pure cultures.

The procedure described in this paper has certain distinct advantages because it is simple, sensitive, quantitative and reliable. However, the reliability of the technique is affected by factors such as differences in Millipore assemblies used during the analysis, variation among technicians, differences in concentration and age of the IgG phosphatase conjugate, length of incubation with the substrate and splitting the experiment over two days. The results obtained serve as a reminder that analytical precision can be expected only if the analytical conditions are constant and carefully controlled.

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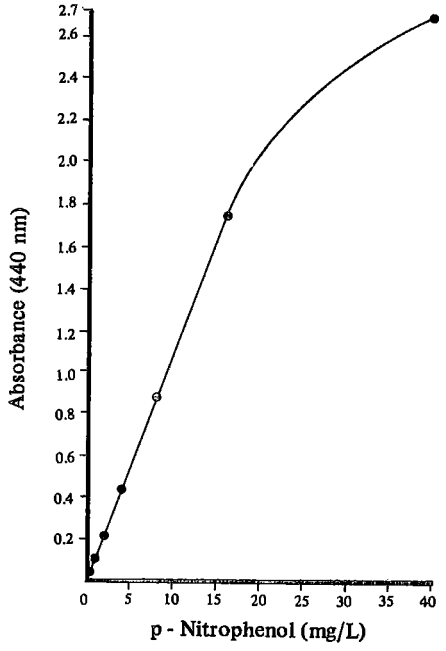

Fig. 1. p-Nitrophenol standard curve.

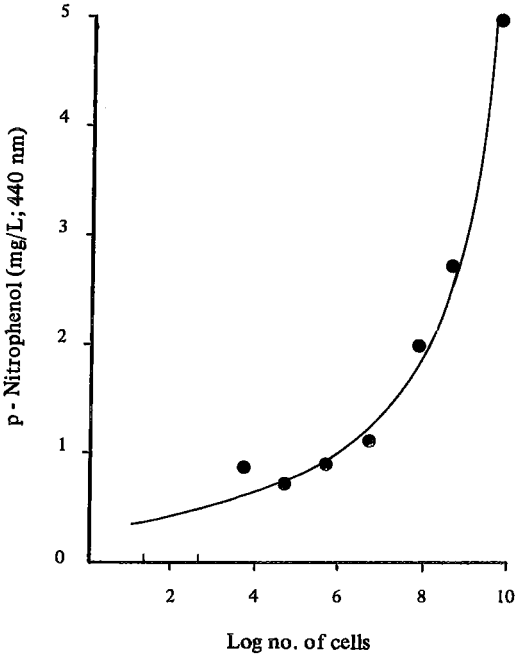

Fig. 2. Relationship between number of Rhizobium japonicum *cells and phosphatase activity at an incubation period of 4h.*

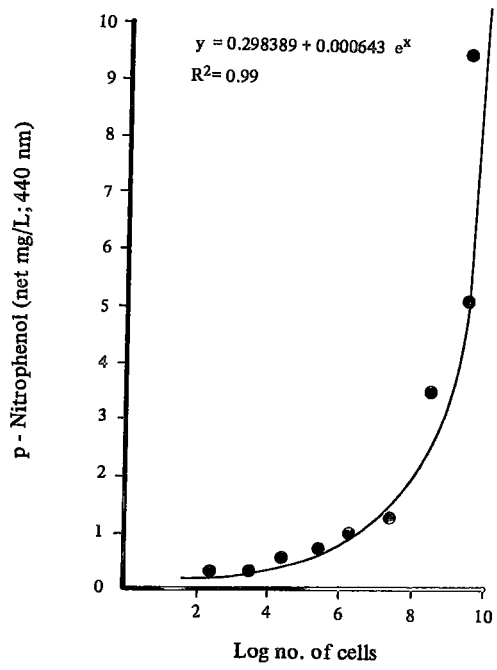

Fig. 3. ***Relationship between number of*** **Rhizobium japonicum cells** ***and phosphatase activity upon increasing enzyme concentration to 2.0 mg/mL.***

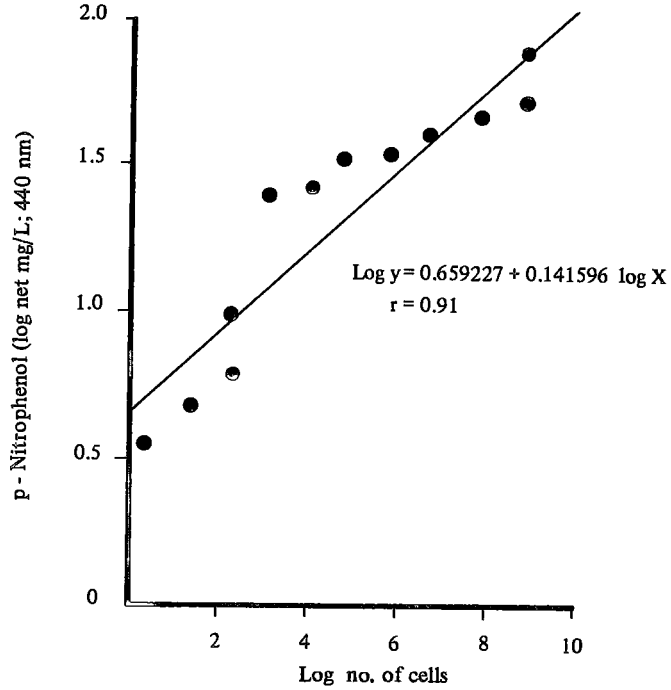

Fig. 4. ***Relationship between number of*** **Rhizobium japonicum cells** ***and phosphatase activity upon conjugation of alkaline phosphatase and IgG with glutaraldehyde for 2 nh.***

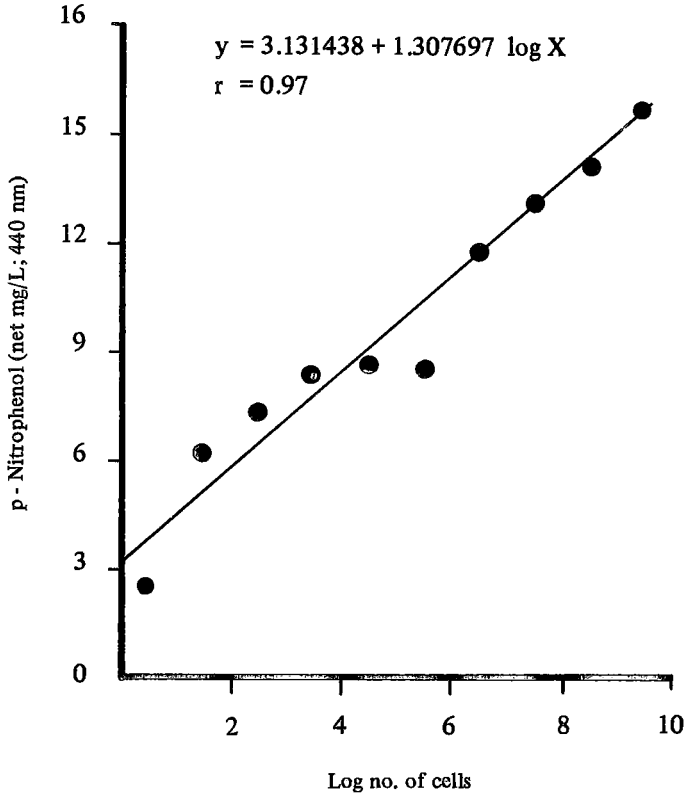

Fig. 5. Relationship between numbers of **Rhizobium japonicum** *and phosphatase activity GA-8 metricel filters for washing rhizobia free of unattached IgG-phosphatase conjugate.*

STUDIES ON RHIZOBIUM ISOLATES FROM TROPICAL LEGUMES OF ECONOMIC IMPORTANCE: I. UTILIZATION OF POTENTIAL ENERGY SOURCES

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ABSTRACT

Effective and less effective strains of rhizobium isolates from *Centrosema pubescens* Benth. (centrosema), C_{11} and C_4; *Leucaena leucocephala* (Lam.) De Wit (ipil-ipil), L_{15} and L_5; *Vigna radiata* (L.) Wilczek, M_5 and M_4; *Arachis hypogaea* L. (peanut), P_3 and P_7; and *Glycine max* (L.) Merr. (soybean), S_{38} and S_{13} were grown in 20 energy sources: arabinose, acetate, citrate, fructose, galactose, glucose, glycerol, inositol, lactate, lactose, malate, maltose, mannitol, mannose, ribose, ribitol, sorbitol, succinate, sucrose and xylose. The Index of Potential Energy Utilization (IPEU) was calculated from the growth responses of the strains to the different energy sources. The IPEU values of the strains are: C_{11}, 0.85; C_4, 0.70; L_{15}, 0.88; L_5, 0.83; M_5, 0.63; M_4, 0.68; P_3, 0.70; P_7, 1.00; S_{38}, 0.88; and S_{13}, 1.00. Effective strains from centrosema and ipil-ipil possess higher IPEU values than the less effective strains while those from cultivated legumes tend to be more discriminating.

The efficiency of utilization of the potential energy source by the rhizobium strains was also calculated. Glucose has the highest and acetate the lowest efficiency of utilization. Mannitol has an intermediate value of 0.75 as compared to 0.90 for glucose and 0.40 for acetate.

INTRODUCTION

There is fierce competition for food among introduced rhizobium, native rhizobium and other soil organisms. One of the criteria for the success of a rhizobium inoculant to survive in the field is to be able to compete successfully for the available food supply in the soil and rhizosphere.

Rhizobia usually can utilize a variety of energy substrates but prefer to grow on simple substrates, the simplest of which is CO_2 which is utilized by *R. japonicum* (Hanus *et al*, 1979) for autotrophic growth. The preferred substrates include sugar alcohols (C_3, C_5, C_6), sugars (C_4, C_5, C_6, C_{12}) (Allen, 1958; Martinez – de Drets *et al*, 1974; Meyer and Pueppke, 1980), organic acids which include acetate, malonate, pyruvate, succinate, fumarate, malate, etc. and amino acids (O'Gara and Shanmugam, 1976; Wilcockson and Werner, 1979). High molecular weight carbon compounds such as cellulose, lignins or

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pectins are hardly utilized by rhizobia (Fahraeus and Ljunggren, 1967). A wide range of phenolic compounds stimulated growth of slow growing rhizobia (Parker, *et al*, 1977). Glen (1981) reported that *R. leguminosarum* 3841 can give good growth in liquid minimal salts medium containing several aromatic carbon compounds.

MATERIALS AND METHODS

Ten rhizobium isolates were obtained from the collection of Dr. E.S. Paterno and Dr. S.N. Tilo of the Department of Soil Science, College of Agriculture, University of the Philippines at Los Baños, College, Laguna (Table 1). The rhizobium cultures were maintained on yeast extract mannitol agar. Transfers were made every month.

The 20 potential energy sources with their corresponding carbon numbers are given in Table 2.

The basal medium (Elkan and Kwik, 1968) in g per L consisted of KH_2PO_4, 1.0; K_2HPO_4, 1.0; NaCl, 0.2; $MgSO_4$, 0.18; $FeCl_3$, 0.04; NH_4Cl,0.5; mannitol 5.0; glutamate, 0.2; $CaSO_4$ (0.1 g/L) was added and any undissolved particles were removed which will interfere in the measurement of optical density of the medium. The pH of the basal medium was adjusted to 7 ± 0.2.

To test each potential energy source, mannitol was replaced in the basal medium by the particular energy source (5 mg energy substrate per mL of basal medium). Five mL of the prepared medium was placed in 25-mL test tubes each plugged with cotton and sterilized at 121°C and 15 lbs per sq inch pressure for 15 minutes.

The inoculum was prepared by growing the particular rhizobium strain in yeast extract mannitol broth for a week and then harvesting the rhizobium cells by centrifugation and gentle suction of the supernatant liquid by a sterile syringe and the residue washed by physiological saline solution. The washed rhizobium cells were then dispersed in physiological saline and its concentration adjusted to give an optical density reading of 0.2. A 0.1 mL of inoculum was introduced into the 5 mL medium containing the specific energy source except for the blanks.

The experiment consisted of 2 replicates per rhizobium strain and 2 blanks (controls) per treatment. Incubation was for 7 days and another set for 5 days at 30°C ± 0.5 in a circulating water bath with intermittent shaking. The growth of the strain was determined by measuring the optical density of the culture medium after the incubation period against a blank in a Bausch and Lomb Spectronic 20 spectrophotometer at a wavelength of 420 nm.

The growth response was rated as follows:

7-day incubation		5-day incubation	
greater than 0.90	4	greater than 0.64	4
0.61 to 0.90	3	0.40 to 0.64	3

0.36 to 0.60	2	0.15 to 0.39	2
0.05 to 0.35	1	0.04 to 0.14	1
0.00 to 0.04	neglible growth (ng)	0.00 to 0.03	ng

Visual observation was used for rhizobium strains with gummy growth. A dense gummy growth was rated 4.

The Index of Potential Energy Utilization (IPEU) of a rhizobium strain was calculated by assigning one point for each energy source in which it scored 4 or 3 and half a point in which it scored 2 or 1. The points were added and the total sum divided by the number of energy sources tested. The value of this ratio would give a measure of the ability of the strain to utilize a variety of potential energy sources. An IPEU value approaching 1.00 would indicate that the strain is capable of utilizing most of the available energy sources in its environment and a value below 0.5 would indicate a very discriminating strain as regards its potential energy source.

The Efficiency Index of the potential energy source was calculated by adding all the scores of the rhizobium strain grown in it and the total sum divided by 40. The number was obtained by multiplying 10 (total number of rhizobium isolates tested) by 4 (maximum growth score). A potential energy source with an efficiency index approaching 1.00 would mean that the energy source could be utilized efficiently by most rhizobium strains and a value lower than 0.3 would be a very poor potential energy source for rhizobium culture.

RESULTS AND DISCUSSION

The growth responses of 10 rhizobium isolates to 20 potential energy sources are given in Table 3. It would be noted that centrosema rhizobium isolates C_4 and C_{11} did not grow well in acetate. Both strains did well in citrate, glucose, malate, maltose, mannose, ribitol and xylose. However, C_{11}, the more effective strain was able to utilize arabinose, fructose, galactose, inositol, maltose, mannitol, mannose, ribose, and sucrose efficiently than C_4.

The rhizobium isolates from ipil-ipil, L_5 and L_{15} can utilize a wide variety of energy substrates quite efficiently except for the organic acids: acetate, citrate, lactate, malate, and succinate. However, L_{15}, the more effective strain, can utilize these organic acids relatively better than L_5, the less effective strain.

The mungbean isolates, M_4 and M_5 exhibited differences in that M_5 was more discriminating than M_4. Both strains grew slower in acetate. Good growth were exhibited by both strains in glucose, glycerol and malate. Intermediate growth rates were shown by M_4 and M_5 in galactose, lactate, ribose, succinate, sucrose, and xylose. However, M_4 grew faster in arabinose, citrate, inositol, mannitol, mannose, and sorbitol while M_5 in fructose and maltose.

The peanut isolates and the soybean isolates show the same trend of the effective strains being more discriminating than the less effective strains. P_7, the less effective

strain grew relatively faster in all 20 energy substrates while P_3, the more effective strain in only 8: citrate, fructose, glucose, lactate, malate, maltose, mannose and succinate. This was also shown by S_{13}, the less effective soybean strain in that S_{13} grew well in all 20 energy substates. S_{38}, the more effective strain did relatively well in 15 energy sources: arabinose, fructose, galactose, glucose, glycerol, lactate, lactose, malate, maltose, mannose, ribose, sorbitol, succinate, sucrose and xylose.

The index of potential energy utilization (IPEU) of a rhizobium strain gives a measure of its ability to utilize a number of potential energy sources. The IPEU values of the rhizobium strains tested are shown in Table 4. The results indicate that effective rhizobial isolates of centrosema, C_{11} and of ipil-ipil, L_{15} have higher IPEU values than the less effective strains, C_4 and L_5. Centrosema is a legume vine which grows wildly and oftentimes used as forage and cover cropping for rubber plantations and other estate crops. Ipil-ipil is a leguminous tree which is used for reforestation and as source of firewood and its young leaves as feed for cattle and poultry. In general, these legumes have to fight for survival in the fields with other plant species and this goes too for their symbiont rhizobia. The survival of their symbiont rhizobia will depend partly on their ability to utilize whatever nutrient sources are available in their environment. However, the more effective rhizobium strains of cultivated legumes, mungbean M_5, peanut P_3, soybean S_{38} have lower IPEU values which may indicate their preference for certain energy substrates. Their host plants may in some manner provide certain of these substrates in the form of root exudates. This may partially explain why there are some host plant preferences by specific rhizobium strains. The effective strains are impelled to invade the roots of their hosts for survival and to escape from the competitive stress imposed by a hostile environment. Thus, effective strains are more infective than less effective ones. There are other factors that determine effectivity (Labandera and Vincent, 1975; Pinto *et al,* 1974; Robinson, 1969a; 1969b) and infectiveness is only one of them. The host plant may orchestrate the selection of one strain over another in the early stages of infection (Bohlool *et al,* 1984).

IPEU values together with other physiochemical properties of the rhizobium strain such as its pH, temperature, oxygen, and moisture level tolerances can help in predicting its survival in the field. Even if the strain survives and multiplies outside the roots of the host plants, if it does not infect the roots of the plant and enter into a symbiotic relationship, then the strain becomes ineffective. The IPEU values give only an indication of the strain's relative growth outside the roots of the host and does not necessarily indicate its effectiveness.

The efficiency by which the potential energy source is utilized by the 10 rhizobium strains is given by its efficiency index as shown in Table 5. Among the 20 energy sources tested, glucose has the highest efficiency index of 0.90 while acetate the lowest with a value of 0.40. Mannitol, the traditional energy source for rhizobial culture has a value of 0.75. Potential energy sources which have higher efficiency indices than mannitol are: glucose, fructose, glycerol, malate, maltose, mannose and xylose. These sugars can replace mannitol in the preparation of culture media for rhizobia and in some cases serve as better sources of energy than mannitol especially for tropical strains.

SUMMARY

Two strains of *Rhizobium* isolates, one more effective than the other, from *C. pubescens* Benth. (centrosema) C_4, C_{11}*; *L. leucocephala* (Lam.) De Wit (ipil-ipil) L_5, L_{15}*; *V. radiata* Merr. (mungbean) M_4, M_5*; *A. hypogaea* L. (peanut) P_3*, P_7 and *G. max* (L.) Merr. (soybean) S_{13}, S_{38}* were tested for their utilization of 20 potential energy sources. To quantify the ability of a strain to utilize potential energy sources, a value called the Index of Potential Energy Utilization (IPEU) was calculated from the results. Effective strains C_{11}, and L_{15} have higher IPEU values than C_4 and L_5 while M_5, P_3, and S_{38} have lower values than the less effective strains M_4, P_7, and S_{13}. This means that the effective strains of field legumes are more discriminating in the utilization of energy sources than those of forest and forage legumes. The IPEU value together with other environmental parameters can help predict the survival of an inoculant strain but may not predict for its infectivity or competitiveness.

The Efficiency Index (EI) of the 20 potential energy sources utilized by the 10 strains was determined. The energy sources were: acetate, citrate, fructose, galactose, glucose, glycerol, inositol, lactate, lactose, malate, maltose, mannitol, mannose, ribose, ribitol, sorbitol, succinate, sucrose and xylose. Glucose has the highest EI, 0.90 while acetate, the lowest, 0.40. Mannitol has an EI of 0.75. Energy sources with EI higher than mannitol are: fructose, 0.80; glycerol, 0.85, malate, 0.78; maltose, 0.88; mannose, 0.85 and xylose, 0.78. These can serve as energy sources of culturing tropical rhizobium in place of mannitol.

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Table 1. Strain designation of rhizobium.

Strain Designation	Host Plant
C_4	*Centrosema pubescens* Benth. (centrosema)
C_{11}*	——— do ———
L_5	*Leucaena leucocephala* (Lam.) De Wit ("ipil-ipil")
L_{15}*	——— do ———
M_4	*Vigna radiata* (L.) Wilczek (mungbean)
M_5*	——— do ———
P_3*	*Arachis hypogaea* L. (peanut)
P_7	——— do ———
S_{13}	*Glycine max* (L.) Merr. (soybean)
S_{38}*	——— do ———

*More effective strain.

Table 2. The potential energy sources utilized by the rhizobial isolates and their carbon number.

Potential Energy Source	Carbon Number
Acetate	2
Lactate, glycerol	3
Malate, succinate	4
Arabinose, ribose, ribitol, xylose	5
Citrate, fructose, galactose, glucose, inositol, mannitol, mannose, sorbitol	6
Lactose, maltose, sucrose	12

Table 3. Growth response of 10 rhizobium isolates from tropical legumes of economic importance to 20 potential energy sources.

Energy Source	Rhizobium Isolate									
	C_4	C_{11}*	L_5[a]	L_{15}*	M_4	M_5*	P_3*	P_7	S_{11}	S_{38}*
Arabinose	2	3	4	4	2	1	2	4	3	3
Acetate	1	1	ng	2	1	1	1	3	4	2
Citrate	4	4	ng	2	4	2	3	3	4	2
Fructose	2	3	4	4	2	3	3	4	4	3
Galactose	2	3	4	4	2	2	2	3	4	3
Glucose	3	4	4	4	3	3	4	3	4	4
Glycerol	3	3	4	4	3	3	2	4	4	4
Inositol	2	3	4	4	2	1	2	4	4	1
Lactate	2	2	1	2	2	2	3	3	4	3
Lactose	2	2	4	4	2	2	2	3	3	3
Malate	4	4	1	2	4	4	3	3	3	3
Maltose	3	4	4	4	1	3	4	4	4	4
Mannitol	2	3	4	4	3	2	2	4	4	2
Mannose	3	4	4	4	3	2	3	4	4	3
Ribose	2	3	4	4	2	2	1	4	4	3
Ribitol[b]	4	4	4	4	2	1	1	4	4	2
Sorbitol	2	2	4	4	3	1	2	4	4	3
Succinate	2	2	2	2	2	2	4	3	4	3
Sucrose	2	3	4	4	2	2	2	3	4	3
Xylose	3	3	4	4	2	2	2	4	4	3

*more effective strain.

[a]L_5 and L_{15} exhibited gummy growth.

[b]results based on 7-day incubation period only.

ng = negligible growth

Table 4. Index of potential energy utilization of 10 rhizobial isolates from tropical legumes of economic importance.

Rhizobium Strain	Index of Potential Energy Utilization
C_4	0.70
C_{11}*	0.85
L_5	0.83
L_{15}*	0.88
M_4	0.68
M_5	0.63
P_3*	0.70
P_7	1.00
S_{13}	1.00
S_{38}	0.88

*more effective strain.

Table 5. Efficiency index of 20 potential energy sources for 10 rhizobium strain isolates from centrosema, "ipil-ipil", mungbean, peanut, and soybean.

Energy Source	Efficiency Index
Glucose	0.90
Maltose	0.88
Glycerol	0.85
Mannose	0.85
Fructose	0.80
Malate	0.78
Xylose	0.78
Mannitol	0.75
Ribitol	0.75
Galactose	0.73
Sorbitol	0.73
Sucrose	0.73
Citrate	0.70
Ribose	0.70
Arabinose	0.68
Inositol	0.68
Lactose	0.68
Succinate	0.65
Lactate	0.60
Acetate	0.40

STUDIES ON RHIZOBIUM ISOLATES FROM TROPICAL LEGUMES OF ECONOMIC IMPORTANCE: II. UTILIZATION OF NITROGEN SOURCES

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ABSTRACT

Rhizobium isolates, C_4, C_{11} from *Centrosema pubescens* Benth. (centrosema); L_5, L_{15} from *Leucaena leucocephala* (Lam.) De Wit (ipil-ipil); M_4, M_5 from *Vigna radiata* (L.) Wilczek (mungbean); P_3, P_7 from *Arachis hypogaea* L. (peanut) and S_{13}, S_{38} from *Glycine max* (L.) Merr. (soybean) were grown in culture media containing 23 types of nitrogen sources. The nitrogen sources used were: NH_4Cl only; NH_4Cl and glutamate; NH_4Cl and 20 amino acids (arginine, alanine, aspartate, asparagine, cystine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophan, threonine, tyrosine, and valine); and NH_4Cl and 19 amino acids (20 combinations of minus one amino acid).

Growth responses based on optical density readings at 420 nm were rated from 0 to 4. L_5, L_{15}, P_7 and S_{13} scored 4 in all nitrogen sources. C_4, C_{11}, M_5 and P_3 scored 0 while M_4 and S_{38} scored 1 in basal medium containing NH_4Cl as the sole source of nitrogen. Addition of glutamate to NH_4Cl improved growth of C_4 from 0 to 2; of C_{11} from 0 to 3; of M_4 from 1 to 3; of M_5 from 0 to 2; of P_3 from 0 to 2; and of S_{38} from 1 to 2. All rhizobium isolates tested scored 4 in basal medium containing NH_4Cl and 20 amino acids.

Effective strains, C_{11}, M_5, P_3 and S_{38} and less effective strains, C_4 and M_4 required available organic nitrogen as well as mineral nitrogen for good growth. L_5, L_{15}, P_7 and S_{13} can grow in a medium containing NH_4Cl as the only source of nitrogen. Glutamate, aspartate, valine, cystine and histidine were stimulatory to growth of the effective strains as well as the less effective strains, C_4 and M_4. P_3 was also stimulated by isoleucine, proline, tyrosine and arginine.

INTRODUCTION

Rhizobia have considerable versality in utilizing nitrogenous substrates (Thorne and Walker, 1936; West and Wilson, 1939; Allen and Allen, 1950). Usually, rhizobia require from 5 to 10 ppm nitrogen for optimum growth (Laird and West, 1938). Neilson (1940) reported that 32 out of 39 amino acids were readily assimilated by *Rhizobium leguminosarum*. Amino acids may stimulate oxygen consumption but may not necessarily serve as good sources of nitrogen for growth (Gau and Kung, 1943). Amino acids can also serve as carbon sources to support growth and provide energy (O'Gara and Shanmugam,

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1976; Parker *et al,* 1977; Wilcockson and Werner, 1979). Purine biosynthesis in rhizobial nodules involves glutamate, glutamine, aspartate, serine and glycine (Schubert and Boland, 1984).

This study was conducted to see how efficiently rhizobium isolates from tropical legumes of economic importance can utilize the available nitrogen sources in the form of NH_4Cl and amino acids. This study will also indicate if there are specific amino acid requirements for the effective and less effective strains.

MATERIALS AND METHODS

The rhizobial isolates used in this study were the same strains as those used in the first study on the utilization of potential energy sources. They are: C_4, C_{11} from *Centrosema pubescens* Benth. (centrosema); L_5, L_{15} from *Leucaena leucocephala* (Lam.) De Wit (ipil-ipil); M_4, M_5 from *Vigna radiata* (L.) Wil. (mungbean); P_3, P_7 from *Arachis hypogaea* L. (peanut) and S_{13}, S_{38} from *Glycine max* (L.) Merr. (soybean).

The amino acids used together with NH_4Cl as sources of nitrogen in this study are given in Table 1. The classification of the amino acids is based on the polarity of the R group or residue because it emphasizes the possible functional roles which the amino acids play in proteins (Conn and Stumpf, 1974).

The basal medium (Elkan and Kwik, 1968) consisted of (in g/L) KH_2PO_4, 1.0; K_2HPO_4, 1.0; NaCl, 0.2; $MgSO_4$, 0.18; $FeCl_3$, 0.04; mannitol, 5.0; and $CaSO_4$, 0.1. Any undissolved $CaSO_4$ particles were removed to prevent possible interference in determining the optical density of the medium.

The nitrogen sources were: (1) NH_4Cl, 0.5 g/L; (2) NH_4Cl, 0.5 g/L and glutamate, 0.2 g/L; (3) NH_4Cl, 0.5 g/L and 20 amino acids (1 mg of each amino acid/100 mL); (4) to (23) NH_4Cl, 0.5 g/L and 19 amino acids (20 combinations of minus one amino acid). The amino acid solutions were prepared by weighing 22 mg of each amino acid and dissolving in 110 mL of basal solution containing no amino acid. A synthetic mixture of the 19 amino acids was prepared by mixing 5 mL of each amino acid solution except for the amino acid to be omitted which was substituted by 5 mL of the basal medium. The pH of all the media prepared was adjusted to 7.0 ± 0.2. Five mL of each of the prepared medium was placed into 25-mL test tubes, plugged with cotton and then sterilized at 15 psi pressure for 15 minutes.

The inoculum was prepared by harvesting week-old rhizobium cells grown in yeast extract mannitol broth by centrifugation, washing with physiological saline, and then suspending the washed cells in physiological saline. The optical density of the suspension was adjusted to read 0.2 absorbance units at 420 nm. Each of the prepared test tubes was inoculated with 0.1 mL of inoculum except for the blanks (controls).

Two replicates were run per rhizobium and per control (blank). Incubation period was for 4 days at 30°C in a circulating water bath. Growth responses were determined by measuring the optical density of the growth medium after incubation at 420 nm

against a blank in a Bausch and Lomb Spectronic 20 spectrophotometer. Visual observation was used to estimate growth of L_5 and L_{15} when these rhizobial strains exhibited dense clumpy growth. The slimy clumps caused interferences in optical density measurements.

Growth responses were rated as follows: greater than 0.20 = 4; 0.10 to 0.20 = 3; 0.05 to 0.12 = 2; 0.01 to 0.04 = 1; lower than 0.01 = 0. A score of 4 was given if a dense clump of growth was shown by the rhizobial isolates.

RESULTS AND DISCUSSION

The growth responses of the rhizobial isolates to 3 kinds of nitrogen sources, NH_4Cl only, NH_4Cl and glutamate, and NH_4Cl and 20 amino acids revealed that growth rates are very much affected by the presence of available organic nitrogen for some of the strains (Table 2).

L_5, L_{15}, P_7 and S_{13} apparently gave a faster rate of growth than the other isolates with only NH_4Cl as the sole source of nitrogen indicating that these isolates or strains have the ability to synthesize the necessary amino acids and biologically active nitrogenous compounds from available mineral nitrogen. On the other hand, C_4, C_{11}, M_4, M_5, P_3 and S_{38} did not show discernible growth after this incubation period in the absence of available organic nitrogen. Addition of glutamate enhanced growth of these strains. Glutamate may have performed an essential role in the nitrogen metabolism of these strains.

When NH_4Cl and 20 amino acids were present in the medium, all strains exhibited faster growth rates as compared to growth rates in NH_4Cl only and NH_4Cl and glutamate. The faster growth rates in this medium may indicate that other amino acids besides glutamate are essential or stimulatory to growth of C_4, C_{11}, M_4, M_5, P_3 and S_{38}.

Results in Table 3 indicate that the amino acids belonging to their respective grouping have a sparing effect on each other. However, C_4 had slower growth in the absence of valine and cystine.

P_3, the more effective strain from peanut had lower growth rates than P_7 and the other strains. P_3 had relatively slower growth in the absence of isoleucine, proline and cystine. M_4 and M_5 also have slower growth rates in the absence of cystine even if methionine was present.

The same lower growth rates for P_3 was evident in the absence of Group B amino acids particularly tyrosine. Not only was the lower growth rate of P_3 evident in the absence of one of Groups A and B . P_3 also showed lower growth rates Group B amino acids particularly tyrosine. Not only the lower growth rate of P_3 evident in the absence of one of Groups A and B amino acids but also in the absence of Groups C and D amino acids, particularly arginine. Glutamate and aspartate have a sparing effect on each other

as shown by the growth responses of the strains in the absence of either one.There was no discernible difference in the growth rates of the rhizobial strains when both were present or when only one of these two amino acids was present. Table 4 gives the essential or stimulatory amino acids for the 10 rhizobial strains tested.

Available organic nitrogen particularly glutamate or aspartate was found to be essential as well as stimulatory to growth of rhizobium strains, C_4, C_{11}, M_4, M_5, P_3 and S_{38}. The more effective strain of peanut, P_3, required certain amino acids for optimal growth while the less effective strain, P_7, did not show any requirement for a particular amino acid provided there was an adequate supply of available mineralized nitrogen. The same observations were also shown for the more effective soybean strain, S_{38}, over the less effective strain, S_{13}. Because of certain limitations of the optical density method for the determination of growth responses, differences in the utilization of nitrogen sources between L_{15}, the more effective strain, and L_5, the less effective strain, were not well defined. L_{15} and L_5 formed thick and slimy growth clumps. Differences between C_{11}, the more effective strain, over C_4, the less effective strain was in terms of the less discriminating nature of C_{11}, C_4 was stimulated by glutamate/aspartate, cystine, and valine while C_{11} required only the addition of available organic nitrogen besides ammonium nitrogen to stimulate growth. The requirement for available organic nitrogen was satisfied by the addition of glutamate/aspartate.

Here, we note as in the previous study on the utilization of potential energy sources that effective strains, with the exception of L_{15} and C_{11}, tend to require certain substrates for optimal growth. This again partly explains why certain rhizobium strains are attracted to the root systems of specific host plants which will provide the necessary stimulatory substances for their survival and growth. Presumably, we can say that less effective strains tested in this study were not under heavy stress to invade and thus infect the roots of their host plant as long as there was an adequate supply of available energy sources and mineral nitrogen. For the more effective strains which require specific amino acids and other growth factors, it was a matter of survival to invade and infect the roots of the host plant for these essential elements. The results of this study tend to suggest that rhizobium strains which require available organic nitrogen besides available mineral nitrogen will be attracted to roots of plants which exude the particular amino acid needed and the other necessary growth factors which are usually vitamins, hormones, and other low molecular weight nitrogenous compounds. These biologically active compounds if present in the root exudates serve as attractants propelling the rhizobia to the surface of the roots of the host plant. Once the rhizobia are on the roots, they will be impelled to seek more food as these root exudates are used up during growth and multiplication of the rhizobia. In the search for more food, they are prodded to invade the roots of their host. Other factors then enter in the infection process which will not be discussed here. Further studies on rhizobium-host plant interaction will either disprove or confirm this attraction theory.

In predicting the survival of the rhizobium strain in the field, the less discriminating strain in terms of its ability to utilize the available energy and nitrogen sources may have better chances of survival outside the roots of the host plant. However, studies by Labaudera and Vincent (1975) showed that the ability of a strain to survive outside the roots of

the host plant is not necessarily related to its competitiveness in nodule formation or effectivity. The results of this study reinforced their observations.

SUMMARY

Rhizobium isolates from *C. pubescens* Benth. (centrosema) C_4, C_{11}*; *L. leucocephala* (ipil-ipil) (Lam.) De Wit, L_5, L_{15}*; *V. radiata* L. Wil. (mungbean), M_4, M_5*; *A. hypogaea* L. (peanut), P_3*, P_7; and *G. max* (L.) Merr. (soybean), S_{13}, S_{38}* were grown in culture media containing different sources of nitrogen. The nitrogen sources are: NH_4Cl only; NH_4Cl and glutamate; NH_4Cl and 20 amino acids; and NH_4Cl plus 19 amino acids (20 combinations of minus one amino acid).

L_5, L_{15}, P_7 and S_{13} were able to grow in a basal medium containing NH_4Cl as the sole source of nitrogen; M_4 and S_{38} showed slight growth while C_4, C_{11}, M_5 and P_3 did now show discernible growth after 4 days at 30°C in this medium. The addition of glutamate and NH_4Cl to the medium enhanced the growth of C_4, C_{11}, M_4, M_5, P_3 and S_{38}. The addition of 20 amino acids and NH_4Cl to the medium enhanced growth further.

L_5, L_{15}, P_7, and S_{13} can survive without organic nitrogen in the medium as long as available mineral nitrogen is present while the rest of the strains need other available organic nitrogen sources. Glutamate, aspartate, valine, cystine and histidine stimulated growth of the effective strains P_3, M_5 and S_{38} as well as the less effective strains C_4 and M_4. P_3 was also stimulated by isoleucine, proline, tyrosine and arginine.

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*more effective strain

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Table 1. Amino acids used and their classification.

Amino Acid	Classification
Group I. Amino acids with non-polar or hydrophobic R groups	
alanine, valine, leucine isoleucine, methionine, cystine, proline	A. Aliphatic (linear, branched and cyclic)
Phenylalanine, tryptophan	B. Aromatic
Group II. Amino acids with polar but uncharged R groups	
serine, threonine, tyrosine (classified as aromatic also)	A. Containing a hydroxyl group
asparagine, glutamine	B. Containing an amide group
glycine	C. Lacks an R group but is definitely polar
Group III. Amino acids with positively charged groups at the physiological pH of 7.	
arginine, lysine, histidine	
Group IV. Amino acids with negatively charged R groups at the physiological pH of 7.	
aspartate, glutamate	

Table 2. Growth responses of 10 tropical rhizobium isolates to three sources of nitrogen, NH_4Cl, NH_4Cl and glutamate, and NH_4Cl and 20 amino acids.

Nitrogen source	Rhizobium Isolate									
	C_4	C_{11}*	L_5	L_{15}*	M_4	M_5*	P_3*	P_7	S_{13}	S_{38}
NH_4Cl	0	0	4	4	1	0	0	4	4	1
NH_4Cl + Glu[a]	2	3	4	4	3	2	2	4	4	2
NH_4Cl + 20 amino acids	4	4	4	4	4	4	4	4	4	4

*more effective strain.

[a]average value of 5-day and 7-day incubation period.

Table 3. Growth responses of 10 rhizobium isolates to NH_4Cl and 19 amino acids (1 amino acid missing out of 20 amino acids used in this study).

Amino Acid Missing	C_4*	C_{11}	L_5	L_{15}*	M_4	M_5	P_3*	P_7	S_{13}	S_{38}*
A. Minus 1 amino acid with nonpolar or hydrophobic R group										
Alanine	4	4	4	4	4	4	3	4	4	4
Valine	2	4	4	4	4	4	4	4	4	4
Leucine	4	4	4	4	4	4	3	4	4	4
Isoleucine	4	4	4	4	4	4	2	4	4	4
Proline	4	4	4	4	4	4	2	4	4	4
Methionine	4	4	4	4	4	4	3	4	4	4
Cystine	3	4	4	4	3	3	2	4	4	4
Phenylalanine	4	4	4	4	4	4	3	4	4	4
Tryptophan	4	4	4	4	4	4	3	4	4	4
B. Minus 1 amino acid with polar but uncharged R groups										
Serine	4	4	4	4	4	4	3	4	4	4
Threonine	4	4	4	4	4	4	3	4	4	4
Tyrosine	4	4	4	4	4	4	3	4	4	4
Asparagine	4	4	4	4	4	4	3	4	4	4
Glutamine	4	4	4	4	4	4	3	4	4	4
Glycine[a]	4	4	4	4	4	4	3	4	4	4
C. Minus 1 amino acid with positively charged R groups at the physiological pH of 7										
Aspartic acid	4	4	4	4	4	4	3	4	4	4
Glumatic acid	4	4	4	4	4	4	3	4	4	4
D. Minus 1 amino acid with negatively charged R group at the physiological pH of 7										
Arginine	4	4	4	4	4	4	2	4	4	4
Histidine	4	4	4	4	3	4	3	4	4	4
Lysine	4	4	4	4	4	4	3	4	4	4

*more effective strains.

[a]lacks a polar R group but amino acid is definitely polar.

Table 4. Essential or stimulating amino acids for 10 tropical rhizobial isolates.

Essential/Stimulating Amino Acid	Rhizobial Isolate
Glutamate/aspartate	C_4, C_{11}*, M_4, M_5*, P_3*, S_{38}*
Cystine	C_4, M_4, M_5, P_3
Valine	C_4
Histidine	M_4
Isoleucine, proline, tyrosine, arginine	P_3

L_5, L_{15}*, P_7, and S_{13} can utilize available ammonium nitrogen and carbon sources for the synthesis of the necessary amino acids.

*more effective strains.

PHOTOCONTROLLED GROWTH, NITROGEN FIXATION AND MORPHOLOGICAL DEVELOPMENTAL LIFE CYCLE IN THE BLUE-GREEN ALGA, *NOSTOC COMMUNE* 45

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ABSTRACT

Growth of the blue-green alga, *Nostoc commune* 45, was determined in 2 sequential light treatments, after first exposure to light when the culture vessels were wrapped separately with various colored cellophanes such as, clear, red and blue and again after a second light sequence when the cellophanes were removed. Better growth was obtained immediately in the control and in clear-cellophaned cultures after the first light exposure. Cultures with red and blue cellophanes showed better growth only after the second sequential light treatment when they were exposed to white light.

The alga exhibited low growth and photodependent acetylene reduction activity (ARA). Blue-cellophaned and dark cultures showed minimum growth and activity and the alga were mostly in aseriate colony and spirally aggregated hormogonia. Better growth and ARA were observed in cultures grown under white light where heterocystous filaments predominate.

The morpho-developmental life cycle of *N. commune* 45 consisted of an alternation of a heterocystous and a sporogenous generation. Expression of the heterocystous generation (nostocacean filaments, motile trichomes or hormogonia and aseriate colony) was greatly dependent on light quality while the sporogenous phase (spore formation and filament anastomosis) was more affected by the presence of exogenous organic carbon compounds.

INTRODUCTION

A common nitrogen-fixing blue-green alga (BGA) in wetland ricefields of the Philippines is the "ball-forming" *N. commune* Vauch, also called "tab-taba" or "bol-boldyok" in the Ilokano dialect.

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Relative abundance of this alga in the paddy field ecosystem may be influenced by the regulatory effect of the rice canopy with regards to amount and type of light affecting its microenvironment. Several evidences have already been presented relating to the importance of spectral qualities on cellular and colonial organization of *Nostoc* species under laboratory conditions (Lazaroff, 1955, 1973; Lazaroff and Schiff, 1962; Lazaroff and Vishniac, 1961, 1962, 1964; Robinson and Miller, 1970). However, no study has yet been done on the effect of light qualities on growth and nitrogen fixation.

Hence, it is of interest to examine the response of this *N. commune* strain to varying light qualities not only on its developmental life cycle but also on growth and nitrogen fixation in the hope of understanding better its eco-physiology.

MATERIALS AND METHODS

Nostoc commune 45 "balls" were collected from a ricefield in Mangatarem, Pangasinan, Philippines and maintained in pure unialgal culture. Various media and cultural conditions were tried mainly to induce the formation of the different morphological stages of the alga.

Media

The basal inorganic medium (G) was adopted from Gerloff *et al* (1950)) and was supplemented either with micronutrients (M) following that of BG-11 medium (Stainer *et al*, 1971) or with soil water extract (SWE; 1:3 w/v Maahas clay in tap water). The SWE was added to the basal medium at an equal proportion (v/v) before autoclaving. As an exogenous organic carbon source, 3 g of glucose was added to 100 mL sterilized G medium. Phosphorous was added as Na_2HPO_4 at twice the concentration of the basal medium or deleted from the medium.

All media were adjusted to pH 7.5 before autoclaving and 15 mL was dispensed in 75 mL test tubes plugged with cotton.

Cultural Conditions

Illumination was provided by 20 W cool, white daylight fluorescent lamps (Philips 54) and 50 W incandescent bulbs (Starlight). The former shows greater energy of emission towards the blue region of the spectrum ranging from 500-550 nanometers (nm). Incandescent bulbs usually have higher spectral energy distribution in the red region, between 600-700 nm (Wright, 1958).

Continuous or interrupted illumination was provided from either a single fluorescent lamp or a fluorescent lamp + 2 incandescent bulbs, or 1 fluorescent lamp + 4 incandescent bulbs that supplied about 2,000, 4,500 and 6,500 lux, respectively. Cultures were placed at a distance of about 17 cm from the lamps. Dark cultures were kept in light tight boxes. Light intensity was measured with a G.E. light meter type # 214.

Experiments were conducted on unaerated liquid cultures in an air-conditioned room (29.0 ± 1.0°C).

Determination of Growth

Growth was determined in algae grown under four light treatments after two light sequences. In the first sequence, different light qualities were provided by wrapping the culture vessels with four layers of either clear (C), red (R), or blue (B) cellophanes and the control (unwrapped). These were removed from the culture vessels for the second light treatment. Both sequences had the same light intensity.

Transmittance curves (Fig. 1) of the cellophanes were established in a Hitachi Model 200-20 scanning spectrophotometer. Clear cellophane absorbed up to about 79% at 190 nm of ultraviolet rays – (190-230 nm). The blue cellophane on the other hand, had comparatively lower transmittance ability for its effective color than the clear and red ones.

Growth studies were done in G + M medium. Cultures were subjected to preconditioning treatment of 16 h light + 8 h dark per day for 7 days prior to growth determination. Growth was measured 3 days after the first exposure, that is, with cellophanes, and 3 days after the second light phase, without cellophanes. Initial algal inoculum before the first exposure was 1 g.

Growth was expressed as specific growth rate (K) that was derived from the equation:

$$K = \frac{2.303\ (\log M_2 - \log M_1)}{t_2 - t_1},\ \text{where K is day}^{-1}\ \text{and}$$

M is fresh weight.

Determination of Nitrogen Fixation

Acetylene reduction assay (ARA) was conducted in 50 mL Erlenmeyer flasks under an atmosphere of 10% acetylene in air. One gram fresh weight algal sample was grown in 10 mL basal medium for a total of 6 days (3 days colored light + 3 days white light). All treatments were the same as those in growth study except for the addition of a dark culture that was kept light tight by wrapping the vessel with 2 layers of carbon paper and keeping in a light tight box.

Acetylene was injected after the second sequential light exposure for each treatment and the cultures were incubated under white light for 24 h at 6,500 lux. Gas samples were removed after 0 and 24 h of incubation and analyzed in a Shimadzu gas chromatograph (GC-7A). Data are presented as nanomoles of ethylene produced per g (dry weight) per hour.

All experimental results (growth and ARA) were averages of 6 replicates per treatment. Results were subjected to statistical analyses using Duncan's multiple range test.

Microscopy and Photomicrography

All microscopic observations of the various growth stages were taken from a piece of *Nostoc* ball that was gently crushed in between the cover slip and glass slide.

Light micrographs were taken with a camera attached on an American Optical Series 10 Microstar laboratory microscope using Kodak panchromatic (Plus-X pan) black and white films.

RESULTS AND DISCUSSION

A. Growth

This strain of blue-green alga exhibited relatively low growth. The highest growth rate obtained was 0.20 g (fresh weight) per day or a doubling period of 5 days. This is about 15x longer than what was earlier reported for another species of *Nostoc, N. muscorum* (Clendenning *et al,* 1956). A possible reason for our strain's low growth response could be the presence of the thick mucilaginous sheath enveloping the ball colony that may be screening some amount and kinds of light and other gaseous substances.

Our data show a relationship between growth and light treatments. Figure 2 shows that after the first light exposure (A) a better growth was obtained among those under clear cellophane and the control. There was as much as 84% increase in fresh weight in the clear cellophaned-cultures in 3 days while the increase in weight for the control, blue and red-cellophaned cultures were 36%, 14% and 6%, respectively. The higher growth noted in the cultures with clear cellophanes over the control (unwrapped cultures) could be due to an additional screening of the lethal, short ultraviolet rays (Fig. 1). On the other hand, a unilateral exposure to either red or blue light did not support relatively good growth because simultaneous exposure to these colors is needed in photosynthesis (Noggle and Fritz, 1977). Minimal activity could occur, but less efficiently, using the phycocyanin and carotenoid pigments (Emerson and Lewis, 1942).

After the second light exposure, when the cellophane wrappings were removed and the cultures were uniformly exposed to white light, those that were previously exposed to red and blue light attained greater increase in weight than after their first light exposure.

A better growth was exhibited by the cultures previously wrapped with red cellophane than with blue implying that red is probably a better photosynthetic color than the latter. This phenomenon was earlier demonstrated in some species of *Chroococcus, Oscillatoria, Anabaena* and *Phormidium* (Emerson and Lewis, 1942; Haxo and Blinks, 1950). Another possible explanation could be the difference in the amount of light transmitted through these cellophanes. Fig. 1 shows that there was about 2x more effective light transmitted through the red cellophane (98%) than in the blue one (68%), so that there was more photosynthetic red color available than blue.

A lesser increase in growth of the control and clear-cellophaned cultures after the second light exposure (B) as compared to the first light exposure (A) may be due to a decrease in available light brought about by concomitant increase in the size of the ball colonies causing self-shading.

B. Nitrogen Fixation

The organism was expected to have low specific activity (ARA) since it exhibited low growth rate. The highest ARA value was 3.21 nmole C_2H_4 produced per

h.g^{-1} (dry weight) under clear-white light treatment (Table 1). This is equivalent to about 2.7 x 10^{-5} nmole ethylene produced per mg protein min $^{-1}$ which is about 2 x 10^5 lower than the average ARA obtained from cultured BGA under fairly optimum conditions, which is 1-10 nmole ethylene produced per mg protein min^{-1} (Stewart, 1973). Cultural conditions may also be suboptimal because even after a 24 h incubation in acetylene there was no multifold enhancement effect on acetylene reducing activity by the organism (David and Fay, 1977).

Table 1 presents ARA values under the different treatments which do not necessarily indicate the optimum values. The trend seems to follow that of growth in which higher activity was obtained in cultures that were subjected initially to clear light followed by the control, the red and blue-cellophaned cultures. A significant minimum activity was observed in cultures initially wrapped with blue cellophanes and those kept in the dark.

C. Polymorphic developmental life cycle

A schematic morpho-developmental life cycle observed in *N. commune* 45 under different spectral qualities in the basal medium (G) and other modification are presented in Fig. 3. Photo-micrographs of the various morphological stages are presented in Figures 4-25.

The organism expressed two generations within its life cycle, a heterocystous and a sporogenous generation (Fig. 3). The heterocystous generation was further subdivided into forms that developed in the dark and in the light. In the dark, we usually observed the development of a cluster of several undifferentiated cells of greater width compactly arranged within a firm, gelatinous sheath, called aseriate colony or packets (depending upon the obvious presence of the gelatinous sheath). Our study confirmed an earlier report that darkness was not essential in the formation of aseriate packets (Lazaroff, 1973) because we observed the same formation under red and white lights (Fig. 4). The aggregated vegetative cells were dark blue-green and measured up to 10 microns (μm) in diameter. The initial stage of aseriate packets was usually confined to lateral divisions of the intercalary vegetative cells in a hormogonium or trichome that caused a proliferation of vegetative cells which appeared as a rosette of cells attached to the original trichome within the mother sheath (Fig. 4). Prolonged incubation up to about 5 days in the dark, caused the increase in number of large subspherical cells forming a mass of subglobose cells without heterocysts (Fig. 5). This morphological differentiation may be initiated by some metabolites excreted in the dark or some metabolites produced in the light but not released.

These packets of undifferentiated cells may resemble the spores of some blue-green algae except that the growth of the former was more extensive and its development into filamentous forms happened through the disruption of the enclosing gelatinous sheath. Exposure of dark cells to white or red light brought differentiation into filament forms characterized by loosening of the gelatinous sheath containing contorted trichomes and detached heterocysts (Fig. 6). These forms were released from the colony when exposed to red light (Fig. 7) but not to white light as in *N. commune* 584 (Robinson and Miller, 1970).

No study was done to determine the shortest time of exposure to red light to initiate filamentation but it was noted that a 60 min exposure could induce bursting of the colony and the release of the motile trichomes (Fig. 7). However, earlier reports noted that an even shorter exposure of 10 min was enough to cause photo-induction in *N. muscorum* A (Lazaroff, 1973). Our cultures usually needed a 3-day exposure to red light to release more motile trichomes. Lazaroff (1973) observed that this was also the number of days required for differentiation in *N. muscorum* A under white light. However, under prolonged darkness, differentiation also occurred (Lazaroff, 1966). This indicates that light may not be essential for the transformation of the aseriate colony.

The photocontrolled differentiation from aseriate into hormogonial form was studied by Lazaroff and Vishniac in 1961 and 1962 and by Robinson and Miller (1970) using *N. muscorum* A and *N. commune* 584, respectively. The action spectrum for induction coincided with the absorption peak of allophycocyanin (Lazaroff, 1966; Lazaroff and Schiff, 1962). The pigment responsible for the reversal of this action (or the transformation from hormogonia into aseriate colony) is now identified as phycochrome c (Bjorn and Bjorn, 1976). Phycochrome c has been definitely established as a non-photosynthetic pigment due to the results of dosage response studies (Lazaroff, 1973). Thus, the phenomenon is not due to a photoconversion of a phytochrome-type photomorphogenetic pigment (Robinson and Miller, 1970, Lazaroff, 1966) nor is it due to the effect of biliprotein synthesis (Scheibe 1972).

The motility of the trichomes was also observed in earlier reports on *N. commune* 584 and *N. muscorum* A but, the motility–promoting substance was unidentified (Robinson and Miller, 1970; Lazaroff and Vishniac, 1961). The substance promoted reciprocal motility in non-motile cultures of both species. Its activity is photocontrolled, it is excreted only from white and red-irradiated cultures, and it promotes motility only in cultures grown in darkness and in red light.

The motile trichomes or hormogonia released from the aseriate colony were usually made up of 4-10 cells. The cells were actively dividing transversely. They were usually barrel-shaped, about 6 μm in diameter, and dark blue-green with prominent granulations at the center of the protoplasts. Detached, spherical, pale yellow-green heterocysts were usually found nearby (Figs. 8-10). In some cases, the terminal cells appeared pointed away from the trichome which might indicate its transitory form to terminal heterocysts.

Figure 9 shows the motile trichomes in the process of aligning themselves side-by-side. This was more evident in cultures with twice the concentration of phosphate. They were later found closely appressed to each other except for the heterocysts which were left behind (Fig. 10). In some instances, exposure to red light caused the grouping of heterocysts separately from the hormogonia. This might be a phototactic response of the organism resulting in an orderly arrangement of the thalli that causes regular "balling". Such positive phototropism has been shown in *Tolypothrix distorta* (Manten, 1948) and *Gloeotrichia natans*. The latter species usually has a definite macrocolony form *in vivo* but exhibits a disorganized mass of filaments *in vitro*.

The appearance of revolving aggregates of hormogonia was observed under blue light (Figs. 11-13). When phosphate, $HPO_4^=$, was absent in the medium, spiral formation involved only a trichome. However, in the presence of phosphate, several trichomes aggregated in spiral form. The heterocysts were initially found attached within the trichomes but they disappeared upon prolonged exposure to blue light (Figs. 12-13). The spiral form may be 500 μm long and 300 μm wide made up of light blue-green vegetative cells that slowly lost their barrel-shaped form under longer exposure to blue light. This spiral aggregate reverted back into short motile trichomes under white or red light. Revolving spiral aggregates were also observed in *N. muscorum* A when cultured in both liquid and solid media. This was viewed as an adjunct to the transfer of genetic material as observed in earlier photomicrographs (Lazaroff and Vishniac 1964).

The short, non-heterocystous hormogonia then alternatively passed through the sporogenous cycle or developed into their normal nostocacean form or heterocystous filament (Fig. 3).

White light promoted the generation of the sporogenous cycle composed of short filaments of 4 cells that later joined end-to-end. This phenomenon was postulated to be some kind of a sexual process of filament anastomosis (Lazaroff and Vishniac, 1962). After such linkages, some spore-like cells were observed that were differentiated from the vegetative cells by having more massive protoplasts (Fig. 14). Caps were sometimes found around the terminal cells or the heterocysts when cultivated in 1-5% glucose solution in G medium (Quimado, pers. comm.). The spores upon germination gave rise to new filaments or germlings and continued their development in the heterocystous cycle.

A normal, heterocystous filament, consisted of long chain vegetative cells and intercalary heterocysts, that reached up to 200 μm long and enclosed by a firm gelatinous sheath. The vegetative cells were light blue-green, barrel shaped and about 5-6 μm in diameter. This was the usual stage observed under white light (Fig. 15). Heterocystous filaments could be derived directly from the aseriate colony (Fig. 16) or from anastomosis of the motile trichomes; while intercalary heterocysts could have arisen from the differentiation of the vegetative cells. The number of heterocysts within a filament may vary according to light quality and intensity (Lazaroff, 1973, Lazaroff and Vishniac, 1962), the presence of glucose, combined nitrogen, or other extrametabolites (Lazaroff and Vishniac, 1962, Lazaroff, 1973; Fogg, 1949).

Exposure to white light resulted in the formation of an envelope around the vegetative cells excluding the terminal heterocysts (Fig. 17). These ensheathed vegetative cells divided parallel to the longitudinal axis of the trichome (Fig. 18-19). The number of cells within the sheath increased progressively (Fig. 20) until internal filamentation of colonies occurred. A series of these ensheathed cells were futher enveloped by a larger expanded gelatinous sheath (Fig. 22), within which cells were found attached to each other in a contorted manner (Fig. 23-24). Later, the trichomes within the sheath were found in indistinguishable form (Fig. 25) this may also result in aseriate colony formation.

It is apparent then, that certain spectral qualities are either directly or indirectly involved in the expression of certain stages in the life cycle of *N. commune* 45.

D. Interrelationship between growth, nitrogen fixation and morphological development

Table 1 summarizes the relationship of the general morphology of *N. commune* 45 to its growth and nitrogen fixing activity under different light qualities. A higher growth was achieved among the red, clear cellophane-wrapped and control cultures, that had mostly normal, heterocystous filaments and hormogonial forms, than the blue cellophane-wrapped ones. Lower growth under blue light supports our observation that in spirally aggregated trichomes there were probably fewer cells exposed to the available light than there were in the filamentous and hormogonial forms. Growth was least in the dark cultures that had more aseriate forms.

Photodependent acetylene reduction activity (ARA) was recorded highest in the clear cellophane-wrapped and control cultures. Higher activity (ARA) among the heterocystous filaments (clear + control) than in the cultures with hormogonia and detached heterocysts (red) means that even if the heterocysts are the sites of nitrogen fixation process, higher ARA could be observed when these specialized cells are within a filament.

Lower ARA in the blue-irradiated cultures further supported our observation that the spirally aggregated trichomes possessed a decreasing number of heterocysts that eventually disappeared. Slight activity (ARA) noted in the dark cultures may be due to heterotropic nitrogen fixation and/or due to the cultures not being homogenously in aseriate forms (without heterocysts).

SUMMARY AND CONCLUSIONS

The expression of the various morphological forms in the life cycle of *N. commune* 45 was dependent on spectral qualities except for the sporogenous stage that was more affected by the presence of exogenous organic carbon sources. It is also apparent that these various growth stages are related to the growth and nitrogen fixing activity of the organism.

In nature, i.e., under wetland ricefields, *N. commune* "balls" are usually larger than under laboratory conditions (Fig. 26). Several attributes can be accounted for this. One of them could be the difference in the amount and types of light available to the alga under the growing rice plant. For example, the light intensity in the field under summer days could be as much as 75,000 lux, and this value is about 15x higher than what we have in the laboratory.

Different spectral qualities may also be manifested under the rice canopy as influenced by the growing rice plant that could enable the organism to express its various growth stages in its life cycle. Thus, at seedling stage of the rice plant there may be maximum transmission of all light colors in the floodwater but as the rice plant increases its number of tillers, the red and blue lights may decrease their penetration under the canopy while the relative energy from the green portion of the spectrum could increase. Therefore, heterocystous blue-green algae that possess red-green photocontrolled heterocystous morpho-developmental life cycle may exhibit more of the aseriate forms; or a delayed formation of filaments and hormogonia under competition for red light. But

photosynthesis may still occur in this condition because of the presence of phycocyanin pigments.

Thus, the growing rice plant may provide the cyclic light stimulus for the synchronous differentiation of the morpho-developmental life cycle of this blue-green alga. This may partly explain its abundance and large macrocolony "ball-like" form in rice paddies.

But this hypothesis, though likely to be occurring, needs to be further investigated.

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Table 1. The effect of different light treatments on the morphology, growth rate and nitrogen-fixing activity after 6 days of N. commune 45*

Initial exposure (with cellophane filter)	Morphology (Dominant)	Growth rate (g. day^{-1}, fresh wt.)	Generation time (days)	ARA nmole $C_2H_4.h^{-1}.g^{-1}$ (dry wt.)
Control (no cellophane)	long, heterocystous filaments	0.0186 a**	54	2.0122 ab
Clear	long, heterocystous filaments and ensheathing of trichomes	0.0053	189	3.2138 a
Red	hormogonia and detached heterocysts	0.0173 a	57	2.0018 ab
Blue	spiral aggregation of trichomes	–	–	0.7872
Dark (no cellophane)	aseriate colony	–	–	0.1836

*Initial exposure = 16 h light (cultures wrapped with cellophane filters) + 8h dark per day under 6,500 lux for 3 days.
Second exposure = without cellophane but same treatment as above for 3 days.
**Figure(s) followed by the same letter(s) are not significantly different at P=0.05 level.

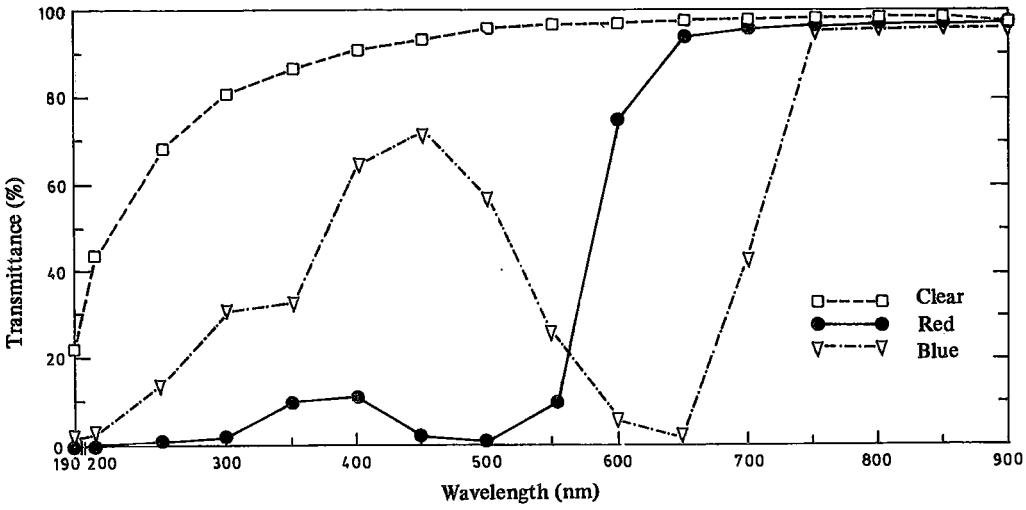


Fig. 1. Spectral transmission of the various cellophane filters.

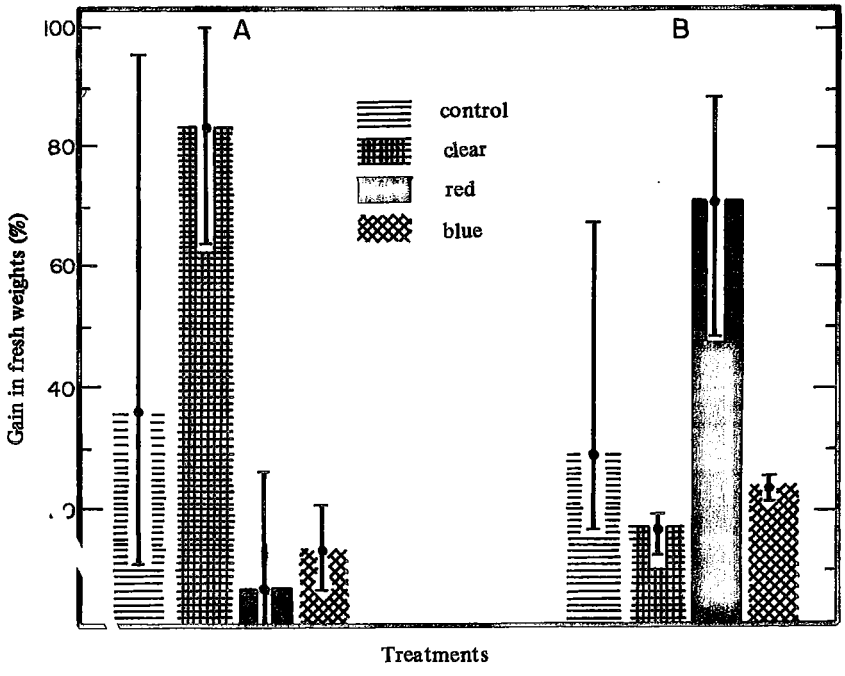


Fig. 2. Gain in fresh weight of N. commune *45 in G & M medium under 2 different sequential light treatments.*

A. Cultures initially exposed for 16 h under different cellophane filters at 4,500 lux + 8 h dark per day for 3 days. Initial weight = 1 g.

B. Cultures grown for another 3 days as A except without cellophanes; Initial weight (g): control = 1.36, clear = 1.84; red = 1.06, blue = 1.14.

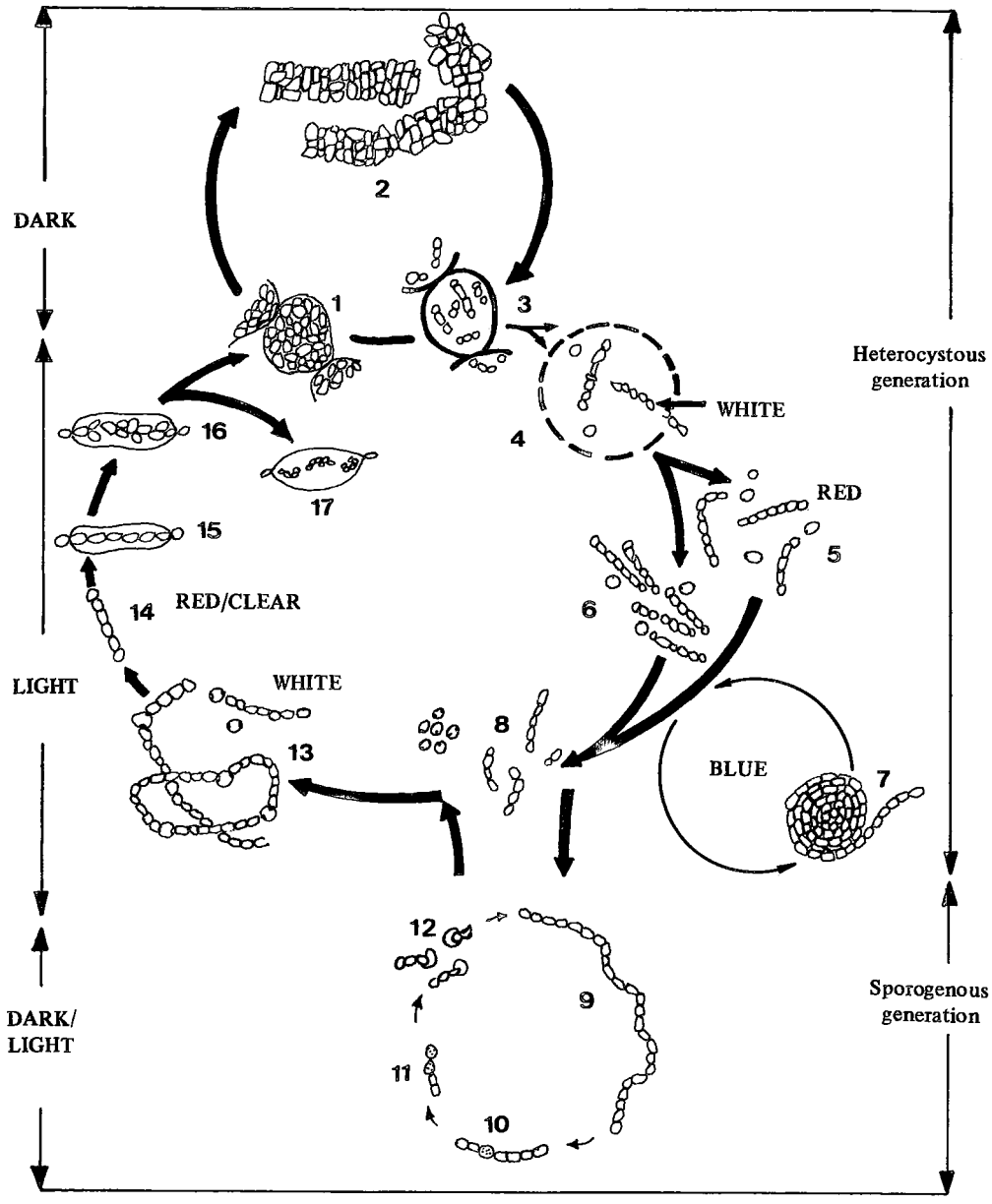


Fig. 3. Diagram of the developmental sequence of the alternation of heterocystous and sporogenous generations in the life cycle of N. commune *45 (see next page).*

A. Heterocystous generation

1. *Aseriate form developed in the dark from the ensheathed trichome in the light.*
2. *Aseriate colonies enclosed by a firm gelatinous sheath.*

3-4 *Trichomes fragmenting at points of attachment of the intercalary heterocysts, loosely arranged within an expanded sheath, later developing into motile trichomes or hormogonia.*

5. *Liberation of motile trichomes from the enclosed sheath usually under red light.*

6, 8 *Alignment of hormogonia side by side, then grouping separately from the detached heterocysts.*

7. *Alternatively, the hormogonia aggregate spirally under blue light.*
13. *Normal, long heterocystous filaments with firm gelatinous sheath, derived either from the sporogenous filaments (9, 10) or short motile trichomes (8).*
14. *Filaments may fragment to form hormogonia again under red or white (clear) light.*
15. *Ensheathment of immobilized hormogonia or trichomes within a loose sheath except at the terminal cells that later develops into terminal heterocysts.*
16. *Successive division of the intercalary cells transversely and horizontally may result into filamentation (13) or formation of several contorted trichomes (17) or aseriate colonies (1-2).*
17. *Several contorted trichomes within a sheath.*

B. Sporogenous generation

9. *Fusion of hormogonia to form long-chain vegetative cells or sporogenous filaments.*
10. *Breaking up of sporogenous filaments into shorter fragments in the absence of heterocysts.*
11. *Spore formation in G+ 3% glucose (w/v) under white light.*
12. *Development of caps around apical cells' external walls in sugar solution.*

Figs. 4-22. Photomicrographs of unstained living materials of N. commune *45 showing stages in its life cycle.*

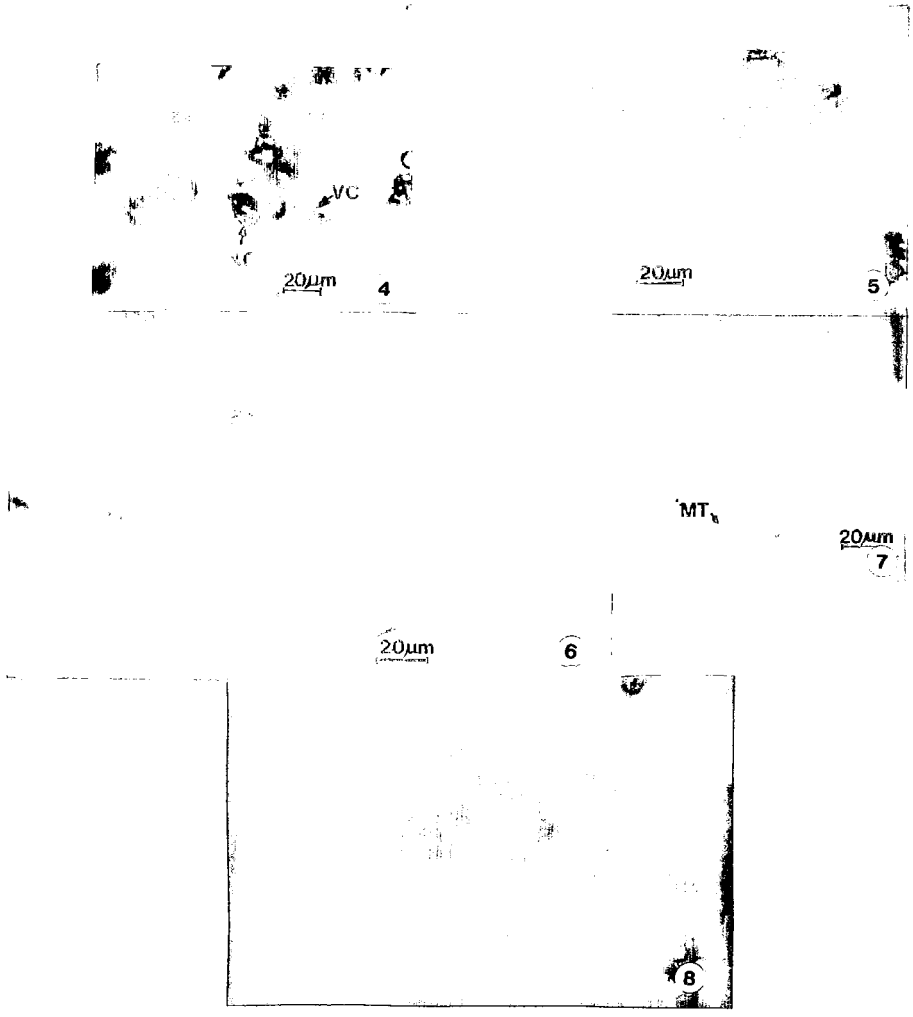


Fig. 4. A young aseriate colony (AC) showing rosette-like cells after a series of division of the vegetative cells (VC) still attached to the original trichome in a loose sheath (S); after 3 d in G + SWE medium under white light at 4,500 lux. (x450).

Fig. 5. An aggregation of several aseriate micro-colonies formed in complete darkness for 5 d in the basal medium. (x450).

Fig. 6. A colonial sheath (S) that expanded showing the loosely arranged short trichomes and detached heterocysts inside that could be derived from an aseriate colony; after 3 d in the basal medium under red light at 6,500 lux. (x450).

Fig. 7. A 60-min exposure to red light at 6,500 lux caused the bursting of the sheath releasing the motile trichomes (MT) or hormogonia in the basal medium. (x100).

Fig. 8. Liberated motile trichomes (MT) above that are in constant motion with detached heterocysts (DH) nearby. (x450).

Figs. 4-22. Photomicrographs of unstained living materials of N. commune *45 showing stages in its life cycle.*

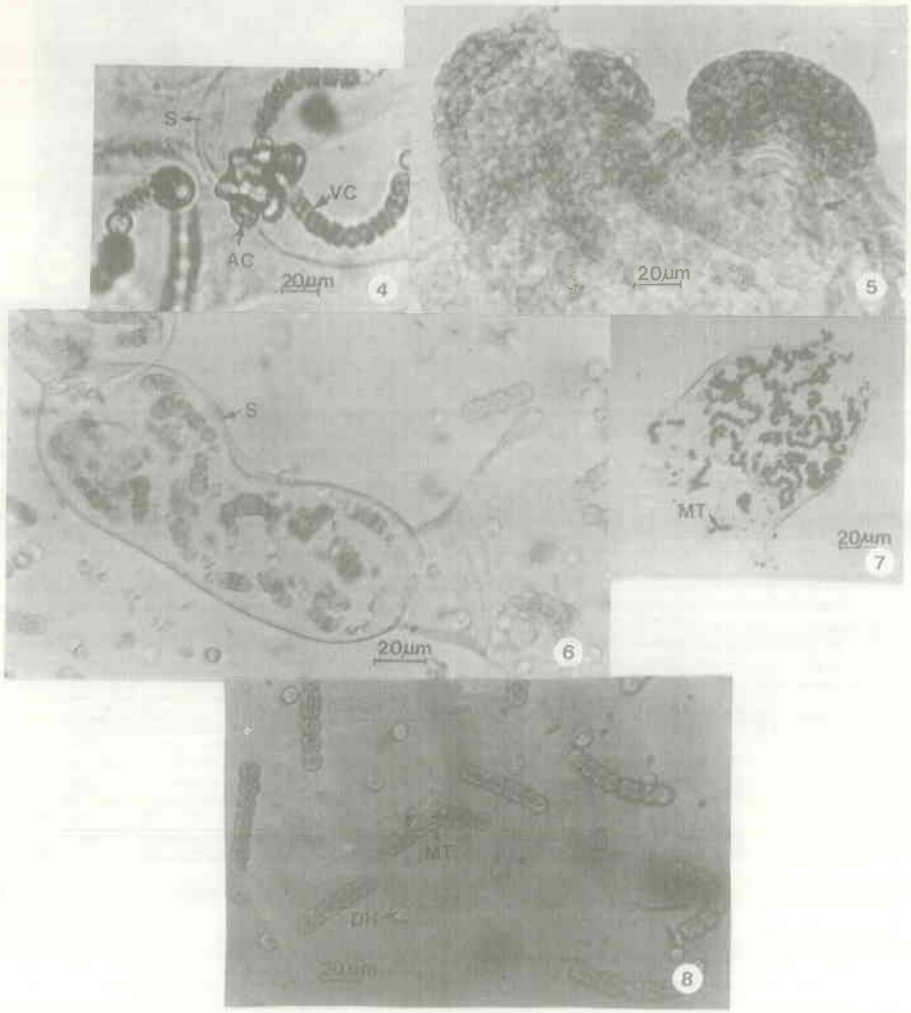


Fig. 4. A young aseriate colony (AC) showing rosette-like cells after a series of division of the vegetative cells (VC) still attached to the original trichome in a loose sheath (S); after 3 d in G + SWE medium under white light at 4,500 lux. (x450).

Fig. 5. An aggregation of several aseriate micro-colonies formed in complete darkness for 5 d in the basal medium. (x450).

Fig. 6. A colonial sheath (S) that expanded showing the loosely arranged short trichomes and detached heterocysts inside that could be derived from an aseriate colony; after 3 d in the basal medium under red light at 6,500 lux. (x450).

Fig. 7. A 60-min exposure to red light at 6,500 lux caused the bursting of the sheath releasing the motile trichomes (MT) or hormogonia in the basal medium. (x100).

Fig. 8. Liberated motile trichomes (MT) above that are in constant motion with detached heterocysts (DH) nearby. (x450).

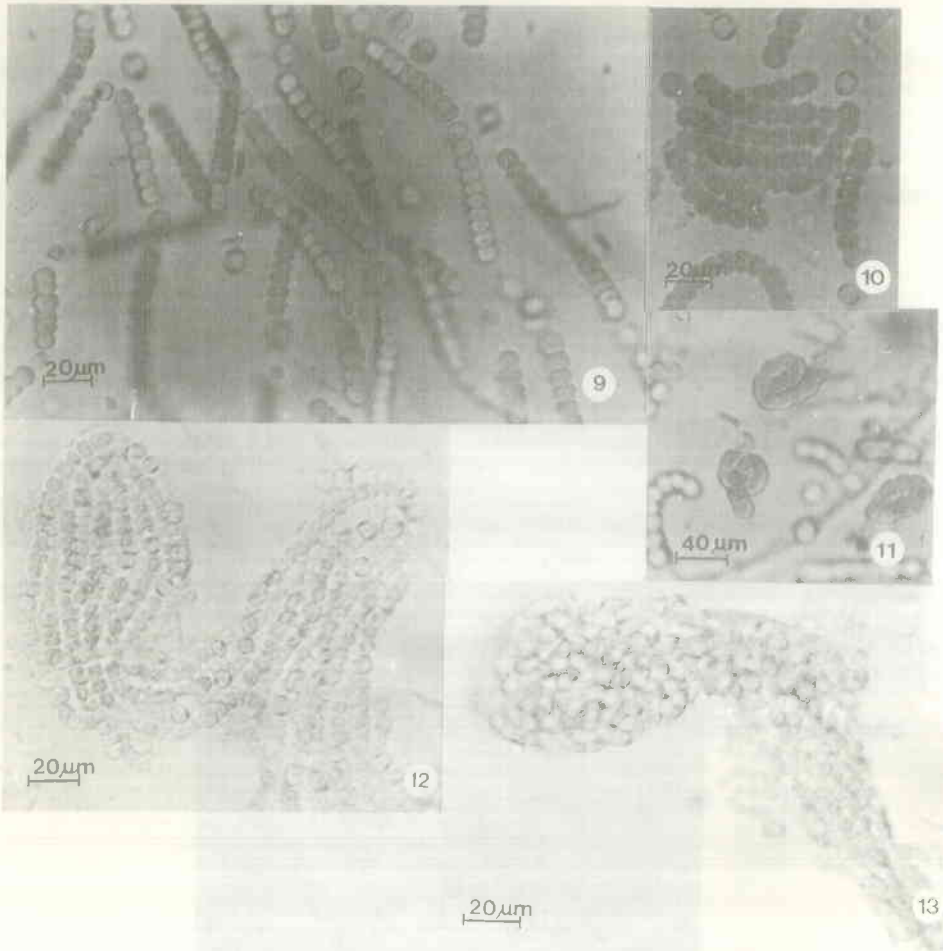


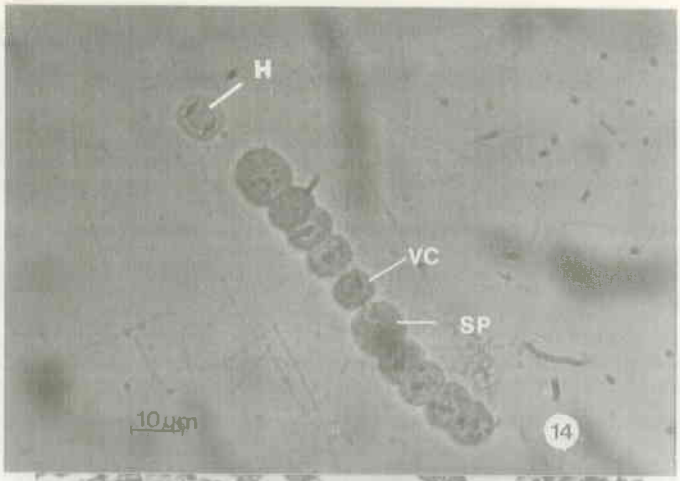
Fig. 9. Alignment of motile trichomes side by side probably as a result of phototactic response; after 6 d in the basal medium + 2 x amount P as Na_2HPO_4 under clear then white light at 6,500 lux (x450).

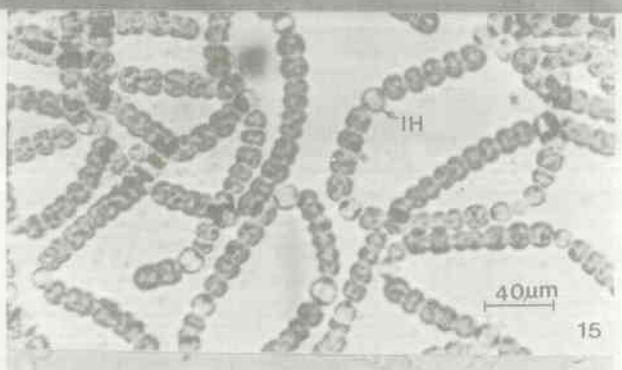
Fig. 10. Separation of hormogonia from the detached heterocysts after 90 min exposure to red light at 6,500 lux in the basal medium. (x450).

Fig. 11. Initial spiral formation of hormogonia exhibited in G medium minus P under blue light at 6,500 lux for 3 d. (x450).

Fig. 12. Spirally aggregated hormogonia with heterocysts observed after 2 h exposure to blue light in the basal medium. (x450).

Fig. 13. Spirally aggregated hormogonia without heterocysts under prolonged exposure (3 d) to blue light at 6,500 lux in G medium + 2x P. (x450).





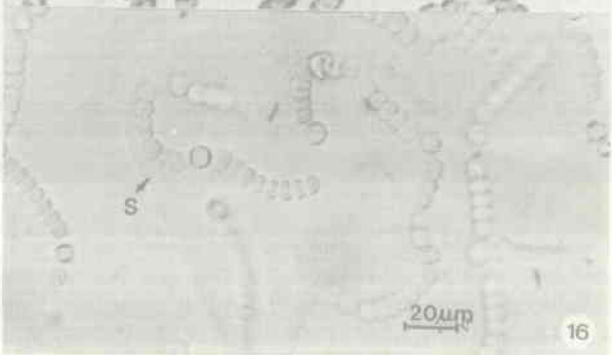


Fig. 14. Spores (SP) differentiated from the vegetative cells (VC) and heterocysts (H) in the fragment by their larger diameter and denser protoplasts; in G + 3% glucose medium under white light for 2 weeks. (x100) (Photo: M. Quimado)

Fig. 15. Formation of normal heterocystous filaments probably from the hormogonia; about 6 vegetative cells in-between intercalary heterocysts (IH) surrounded by a firm, non-evident sheath; 3-d culture under white light at 6,500 lux in the basal medium. (x450).

Fig. 16. Heterocystous filaments could also arise from the aseriate colony as shown here with the loose sheath (S) still attached; 5-d culture grown in the dark then transferred to 3 d under white light at 6,500 lux in the basal medium. (x450).

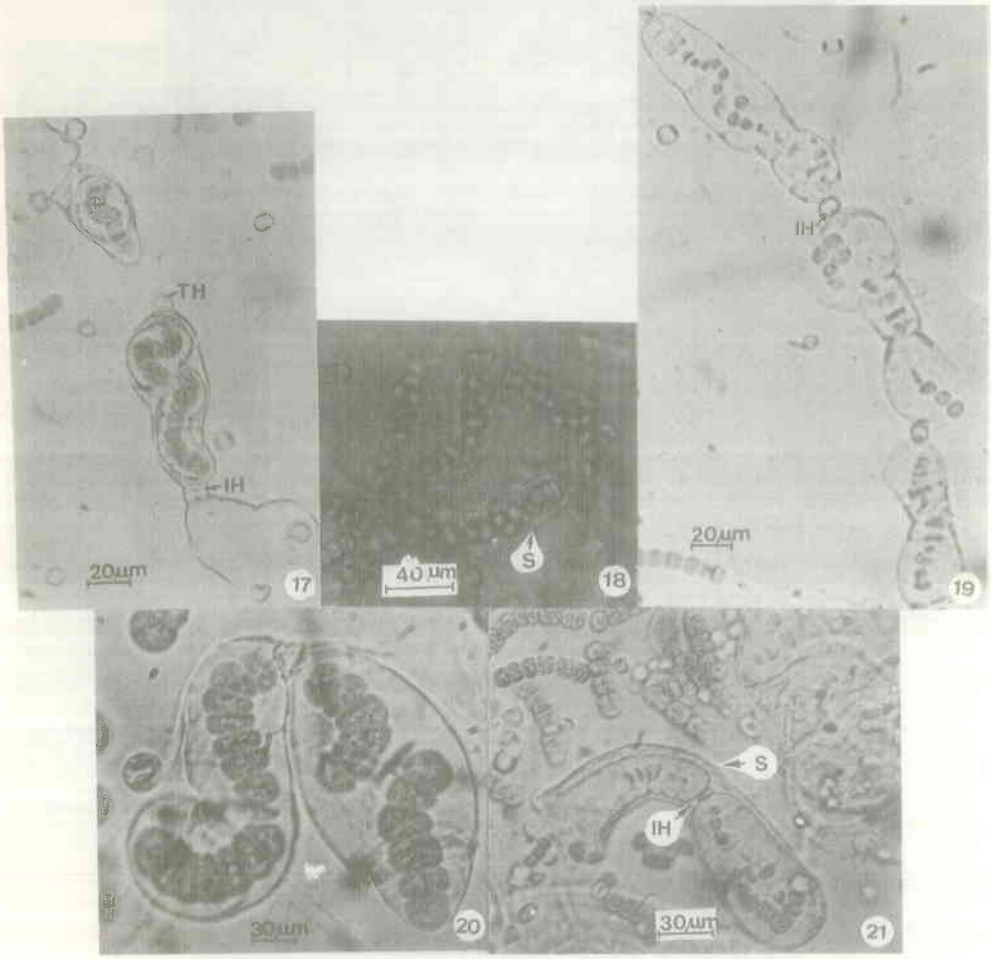


Fig. 17. Two ensheathed trichomes within another common envelope except around the terminal and intercalary heterocysts (TH, IH); after 2 h exposure to clear light at 6,500 lux in the basal medium. (x450).

Fig. 18. Cells actively dividing parallel to the longitudinal axis of the trichome within an expanded sheath (S); after 90 min exposure to white light at 6,500 lux in the basal medium. (x450).

Fig. 19. Perfect pairing of 4 cells probably after reduction-division; intercalary heterocysts (IH) not within the sheath; after 3 d in the basal medium under red light at 6,500 lux. (x450).

Fig. 20. Ensheathed trichomes (6-8 cells) within an ellipsoidal sheath separated from another set by intercalary heterocysts 3-d culture exposed to clear light at 6,500 lux in basal medium. (x1000).

Fig. 21. Filamentation of cells showing a sheath (S) enveloping ensheathed trichomes and the intercalary heterocyst (IH): After 30 min exposure to blue light at 6,500 lux in the basal medium. (x450).

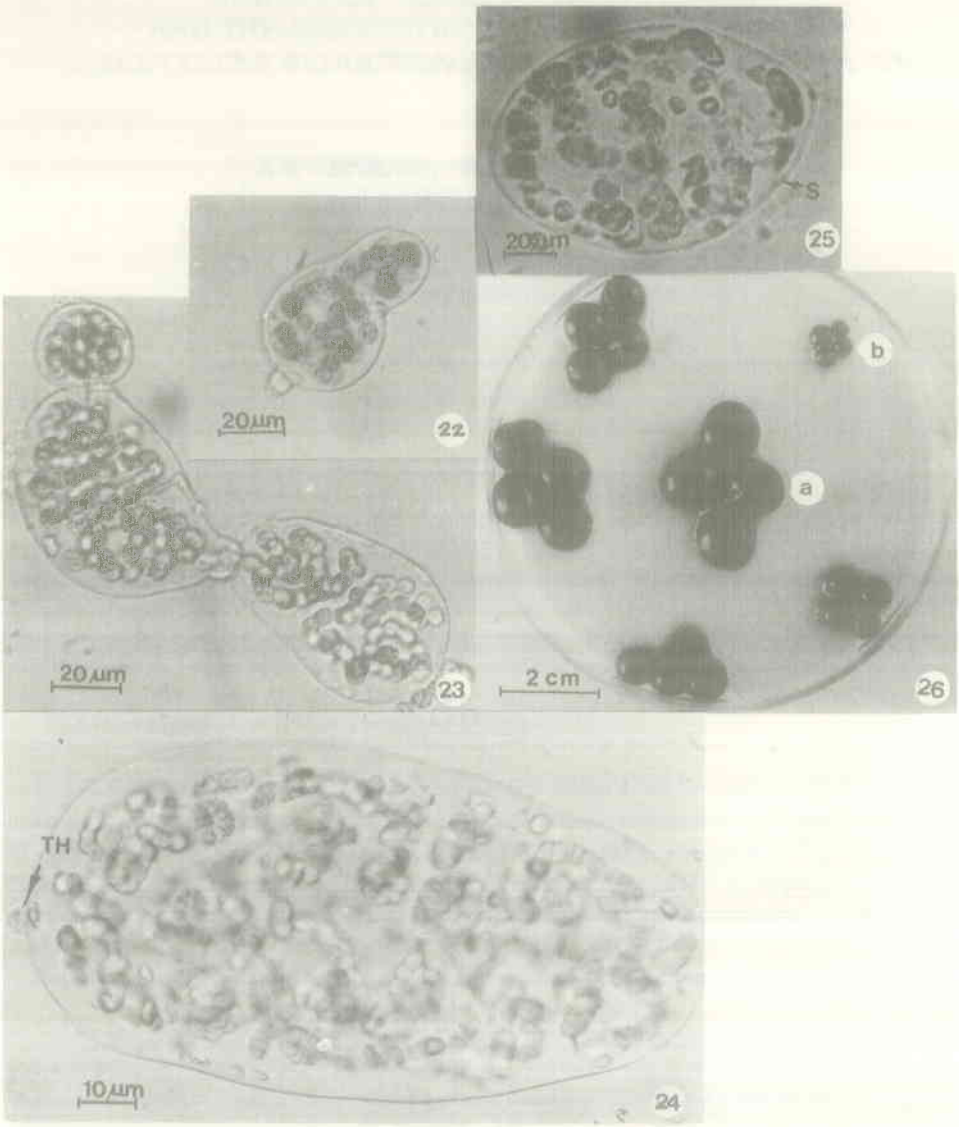


Fig. 22-23 *Instead of filamentations the trichomes in Fig. 17 may keep on dividing to form long chain contorted trichomes within an expanded sheath except at the terminal and intercalary heterocysts; after 3 d in the basal medium under red light at 6,500 lux. (x450).*

Fig. 24. *An expanded colony of contorted trichomes within a sheath showing the terminal heterocyst (TH) outside the colony; after 1 h exposure to red light at 6,500 lux in the basal medium (x450).*

Fig. 25 *An initial stage of the aseriate form within a "ball-like" sheath (S). (x450).*

Fig. 26. Nostoc *balls* in vivo *(a) and* in vitro *(b). Note the differences in size.*

SURVEY OF FLORA OF TAAL VOLCANO AND THE ASSOCIATION OF NITROGEN FIXATION (ACETYLENE REDUCTION ASSAY) WITH CERTAIN PLANTS

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ABSTRACT

A floristic survey of Taal Volcano was conducted to determine the relationship between nitrogen fixation and the establishment of plant species in the area over the last fifteen years. The flora was composed mainly of legumes with well developed nodules and many non-legumes that showed positive nitrogen fixation activity (acetylene reduction assay) in the roots. It is concluded that the diazotrophs in the roots hold the key to the pioneering ability of these plants in such inhospitable environment.

INTRODUCTION

A floristic survey of a volcanic area a few years after its eruption is important in forming an ecological understanding of its vegetational development. In such survey, one is confronted with an intriguing question of why a particular group of plant species is able to colonize more successfully than others. This paper offers an explanation in terms of the ability of the plant to fix nitrogen which in the case of legumes, the hosts enter into a symbiotic relationship with the appropriate *Rhizobium* sp. while in the case of the others, some other bacteria may be involved in an associative type of relationship.

In a survey of Taal Volcano six years after its violent eruption in 1911, Brown *et al.* (1917) reported seeing 292 species of plants representing 232 genera and 66 families. Of these, only 13 species were listed as very common and widely distributed in the area. These were: *Acacia farnesiana* (L.) Willd. (Mimosaceae), *Pithecellobium dulce* (Roxb.) Benth. (Mimosaceae), *Eugenia jambolana* Lam. (Myrtaceae), *Ficus hauili* Blanco, now called *F. septica* Burm. *f.* (Moraceae), *F. indica* L. (Moraceae), *Morinda bracteata* Roxb. (Rubiaceae), *Trema orientalis* Blume (Ulmaceae), *Tabernaemontana subglobosa* Merr. (Apocynaceae), *Antidesma ghaesembilla* Gaerth., now called *A. frutescens* Jack (Euphorbiaceae), *Callicarpa blancoi* Rolfe (Verbenaceae), *Ipomoea pes-caprae* (L.) Roth. (Convol-

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vulaceae), *Bulbostylis barbata* (Rottb.) C.B. Clarke (Cyperaceae) and *Saccharum spontaneum* L. (Gramineae). In another survey of the same area conducted after 50 years of vegetational development, Pancho (1967) enumerated 399 species representing 293 genera and 91 families or an increase of over 100 additional species representing 61 new genera and 25 families. However, of the 13 most common species observed by Brown, *et al*, only *P. dulce, A frutescens, I. pes-caprae* and *S. spontaneum* were observed still common in 1965 while the others were either present sparsely or absent completely but were replaced by some other 113 plant species that became plentiful in the area.

It is noted that the species enumerated in these surveys were also common in the mainland and the seeds were distributed mainly by birds except for others distributed by water such as *I. pes-caprae,* or by wind such as *B. barbata* and *S. spontaneum.* The close proximity of the island to the mainland was partly responsible for its relatively fast revegetation. All other factors considered, it would seem that the pioneer species were replaced by others in a form of vegetational succession.

One week after the Taal volcano eruption in 1965, Pantastico *et al* (1965) tested the fertility status of the volcano ash with tomato as indicator plant. Their findings indicate that the volcano ash deposit was severely deficient in available forms of N, P and K. It seems that for the pioneers to survive, they would have to satisfy their nitrogen requirement, possibly through the biological fixation process. Results are presented here to support nitrogen fixation in their roots with the appropriate diazotrophs as a common characteristic among pioneer species.

MATERIALS AND METHODS

Taal Island is an active volcano located at latitude 14°02′ north and longitude 120°59′ east in Taal Lake, Batangas Province approximately 63 km southwest of Manila, Philippines. In its most recent eruption in September 1965 the heaviest deposition of volcanic ash occurred in the Island's western section bounded by Gunao Pt. and Mataas-na-longos Pt. This served as the study area which was considered ideal for our purpose because of the total destruction of the vegetation in the area and its relative accessibility.

The floristic survey was conducted on June 6 and August 9, 1980. The survey was confined to an area approximately 20 m wide starting from the huge volcanic rocks at the coastal area going up to the rim of the crater and then down to the crater floor.

The area was divided into three distinct sections such as: *A*, the area covered by big volcanic rocks deposited at the edge of the lake, *B*, the area covered by the deep deposit of loose volcanic ash from the base of big volcanic rocks to the rim of the crater representing the highest elevation of the transect (∿150 m above sea level); and *C*, the crater floor approximately 100 m below the rim laden with volcanic cinders (Fig. 1).

Plant samples taken from the area were identified by comparison with the herbarium specimens at the UPLB Museum of Natural History. Root samples were placed in plastic bags and processed in the laboratory for acetylene reduction assay (Aspiras *et al,* 1980).

RESULTS AND DISCUSSION

1. Floral Survey

In the rocky section of the sampling area, the families Moraceae and Pteridaceae were represented by five species each while the others had a single species each (Table 1). Quite prominent in the area were *Ficus* spp., *Pipturus arborescens, Morinda citrifolia, T. orientalis, Syzygium cuminii, Macaranga tanarius* and *Terminalia catappa* and *S. spontaneum. I. pes-caprae* was likewise prominent in rocks close to the lake. The rest of the plants were observed rarely.

In the expanse from the base of the big rocks to the rim of the crater, only four families were represented namely, Gramineae, Papilionaceae, Mimosaceae and Cyperaceae. *S. spontaneum* was the single most widely distributed species. Others were locally abundant such as *Canavalia microcarpa* and *Desmodium scorpiurus* which were quite common as large colonies in the lower portion of the crest. *D. triflorum* were found in clusters around the base of *S. spontaneum* at the lower portion of the crest, *S. cannabina, A. farnesiana, P. dulce* were more frequent at the lower slopes while *Albizia procera* and *Leucaena leucocephala* were more widely distributed throughout this section. The other grasses were found rarely. *Bulbostylis barbata,* a sedge, was widely distributed like *S. spontaneum* but not as common.

At the basin of the crater, families Gramineae, Pteridaceae, Mimosaceae, Compositae and Cyperaceae were represented by at least two species each. However, *S. spontaneum* was still the single most prominent species with occasional legumes and trees such as *M. citrifolia* and *S. cuminii,* the latter trees preferring to grow in volcanic cinders in the rockband along the lake. The ferns were quite common in crevices of rocks. *Fimbristylis dichotoma* and *B. barbata* were widely distributed among clusters of *S. spontaneum* at the lower portion of the basin covered with volcanic soil. The rest of the plants in the list were encountered rather rarely.

It is apparent from the survey that the rocky portion and the crater basin were represented by a more diverse plant population than the crest going up the rim of the crater. Throughout the area *S. spontaenum* was the most common and widely distributed species. Two years after the Taal Volcano eruption in 1911, Gates (1974) observed that the slopes up to the crater were generally vegetated first with grasses followed by shrubs and small trees. In small tree formations, a number of families were represented particularly the Euphorbiaceae, Leguminosae (all of the legumes were placed under the family), Moraceae and the Apocynaceae.

Doctor van Leeuwen (1936) observed a gradual succession of plants in Krakatau after the complete devastation of its vegetation in 1883. The area was invaded first by *S. spontaneum* and *Imperata arundinacea* Cyr. followed by trees that could survive in exposed places such as *M. tanarius, Pipturus incanus* Wedd., and *Ficus* spp. *Casuarina equisetifolia* L., a nodulated non-legume, was quite common in the lower sandy slopes.

Because of its predominance in Taal Volcano *S. spontaneum* was expected to contribute to the organic matter build-up through time. The results of analyses indicate tremendous improvement in nitrogen and organic matter content of the soil in the presence of *S. spontaneum* (Table 2). The pH likewise showed improvement. These results were corroborated by the data of Brown *et al* (1917) who determined the total N and organic matter content of samples taken at the crater slopes and at the grass area and obtained 0.01 and 0.02% total N and 0.37 and 0.38% organic matter, respectively. Higher organic matter values obtained in the area of the present study could be attributed to the extensive root system of *S. spontaneum* since the sample was taken approximately two meters away from the base of a large *S. spontaneum* cluster. Exposed roots of *S. spontaneum* in eroded areas go beyond two meters from its base (Fig. 2). Deep and extensive root systems were generally observed among plants growing in the island specially those located in deep volcanic ash deposits.

2. Acetylene reduction assay (ARA)

The observation that certain plants were able to build up their biomass in the study area in spite of the very low soil fertility level led us to determine the extent of nitrogen fixation (ARA) in the roots of these plants. There was no doubt that the legumes were actively fixing nitrogen because of the well-formed and healthy nodules observed in their roots (Fig. 3) and the nodules were abundant in all the legumes examined. Crushed nodules revealed the presence of a pinkish sap attributable to leghemoglobin which was normally produced in nodules supporting active nitrogenase activity. We would like to mention that this paper is the first known report on nodulation of *Canavalia microcarpa* based on the book of Allen and Allen (1981).

The effectiveness by which *S. spontaneum* invaded extensively open spaces in Taal Volcano could be explained partly by its ability to support nitrogen fixation by the associated bacteria in its roots. Results of ARA showed substantial N_2 fixation by excised roots (Table 3). Nitrogen fixation in the roots of the *Saccharum* species has already been reported (Dobereiner *et al,* 1972). It is no wonder the organic matter was greatly increased in its presence (Table 2). Other plants endowed with similar ability to support nitrogen fixation in the roots include *P. arborescens, T. orientalis, M. tanarius, M. citrifolia* and *Ficus* sp. These were trees found growing mainly in crevices of volcanic rocks. The other trees found in this area were not included in the assay because of our failure to obtain enough root samples. *B. barbata* and *F. dichotoma,* sedges belonging to Family Cyperaceae and widely distributed in the area, likewise showed nitrogenase activity. Additionally, *S. spontaneum, B. barbata* and *F. dichotoma* normally produce abundant seeds that are dispersed by air, resulting in very rapid distribution. Roots of ferns such as *P. vittata* and *O. siliculosum,* likewise showed nitrogenase activity.

The wide variability of the values reported is normally expected in assay of field-collected samples (Aspiras *et al,* 1980; 1981) and the readings obtained in some rhizosphere soil resulted from the difficulty in obtaining soil samples free of fine root hairs.

Although the plants encountered in this survey had not all been assayed for nitrogenase activity, the information herein presented is sufficient to support the contention

that the nitrogen-fixing ability is a major reason for the successful pioneering of these plants in the volcano island. This observation adds a new dimension to our ecological understanding of vegetational development specially in nutrient-poor, open areas. These findings offer a big challenge for us to look into the various diazotrophs involved and determine the mechanism of nitrogen fixation in each case.

SUMMARY

A floristic survey was conducted on the section that was devastated completely by Taal Volcano's most recent eruption fifteen years ago upon which the selection of the plants to be used for nitrogenase activity (acetylene reduction) determination was based. Although not all the plants included in the survey were assayed for nitrogenase activity, those tested were all positive for nitrogen fixation. This confirms our hypothesis that the pioneering ability of a plant in nutrient-poor environment depends on its ability to generate its nitrogen requirement via the biological nitrogen fixation process. It would be of interest therefore to identify the diazotrophs specifically among the non-leguminous species and determine the mechanism of nitrogen fixation involved.

ACKNOWLEDGEMENT

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Table 1. Floral composition of approximately 20-meter wide strip along the slopes of the western section of Taal Volcano.

Family and Species	Number of Species
Section A – Area covering the rockband of volcanic cinders from the lake water level to the base of volcanic ash deposits.	
Family Moraceae	
Ficus cumingii Miq. *F. indica* L. *F. odorata* (Blco). Merr. *F. septica* Burm. f. (= *F. hauili* Blanco) *F. ulmifolia* Lam.	5
Family Pteridaceae	
Adiantum philippense L. *Pityrogramma calomelanos* (L.) Link *Pteris ensiformis* Burm. *P. vittata* L. *Pteris* sp.	5
Family Davalliaceae *Nephrolepis bisserata* (Sw.) Schott	1
Family Sinopteridaceae *Onychium siliculosum* (Desv.) Christ.	1
Family Papilionaceae *Canavalia microcarpa* (DC.) Piper	1
Family Urticaceae *Pipturus arborescens* (Link.) C.B. Rob.	1
Family Rubiaceae *Morinda citrifolia* L.	1
Family Ulmaceae *Trema orientalis* Blume	1
Family Myrtaceae *Syzygium cuminii* (L.) Skeels	1
Family Euphorbiaceae *Macaranga tanarius* (L.) Muell.-Arg.	1
Family Combretaceae *Terminalia catappa* L.	1
Family Gramineae *Saccharum spontaneum* L.	1
Family Convolvulaceae *Ipomoea pes-caprae* (L.) Roth.	1

Table 1 Continued

Family and Species	Number of Species
Family Compositae	
Mikania cordata (Burm.) B.L. Robins	1
Family Verbenaceae	
Lantana camara L.	1
Family Sterculiaceae	
Sterculia foetida L.	1
Sub-total	24
Section B – area covered by deep but loose volcanic soil starting from the base of tall band of rough volcanic cinders up to the rim of the crater.	
Family Gramineae	5
Brachiaria distachya (L.) Stapf (= *Panicum distachyum* L.)	
Dichanthium annulatum (Forsk.) Stapf	
Digitaria ciliaris (Retz.) Koch	
Panicum repens L.	
Saccharum spontaneum L.	
Family Papilionaceae	4
Canavalia microcarpa (DC.) Piper	
Desmodium scorpiurus (Sw.) Desv.	
Desmodium triflorum (L.) DC.	
Sesbania cannabina (Retz.) Pers.	
Family Mimosaceae	4
Acacia farnesiana (L.) Willd.	
Albizia procera (Roxb.) Benth.	
Leucaena leucocephala (Lam.) de Wit	
Pithecellobium dulce (Roxb.) Benth.	
Family Cyperaceae	2
Bulbostylis barbata (Rottb.) C.B. Clarke	
Fimbristylis dichotoma C.B. Clarke	
Sub-total	15
Section C – crater basin	
Family Gramineae	5
Brachiaria distachya (L.) Stapf (= *Panicum distachyum* L.)	
Digitaria ciliaris (Retz.) Pers.	
Panicum repens L.	
Rhynchelytrum repens (Willd.) C.E. Hubb.	
Saccharum spontaneum L.	
Family Pteridaceae	3
Adiantum philippense L.	
Pityrogramma calomelanos (L.) Link	
Pteris vittata L.	

Table 1 Continued

Family and Species		Number of Species
Family Mimosaceae		3
Acacia fernesiana (L.) Willd.		
Leucaena leucocephala (Lam.) de Wit		
Pithecellobium dulce (Roxb.) Benth.		
Family Papilionaceae		1
Canavalia microcarpa (DC.) Piper		
Family Compositae		3
Mikania cordata (Burm.) B.L. Robins		
Tridax procumbens L.		
Vernonia cinerea (L.) Less.		
Family Cyperaceae		2
Bulbostylis barbata (Rottb.) C.B. Clarke		
Fimbristylis dichotoma C.B. Clarke		
Family Rubiaceae		1
Morinda citrifolia L.		
Family Myrtaceae		1
Syzygium cuminii (L.) Skeels		
Family Apocynaceae		1
Ichnocarpus colubilis (Lour.) Merr.		
Cecropiaceae		1
Cecropia peltata L.		
	Sub-total	21
	TOTAL	60

Table 2. Some characteristics of the volcanic ash deposit taken at two sites.

Sampling site	% N	% OM	pH
Base of *S. spontaneum*	0.08	1.34	6.5
Bare area (first 10 cm)	0.02	0.52	3.4

These data were provided by the Department of Soil Science Analytical Laboratory.

Total N was determined by the micro-Kjeldahl procedure, organic matter by chromic acid digestion procedure of Walkley and Black and pH with the use of Beckman pH meter at 1: 1 soil-water ratio.

Table 3. Nitrogen fixation (acetylene reduction) in roots of plants growing in volcanic soil and in the crevices of volcanic rocks.

Species	nmoles C_2H_4/g fresh wt/24 h	
	roots	*rhizosphere soil*
Saccharum spontaneum	113.3 ± 47.6[a]	0
Pipturus arborescens	8.5 ± 6.0	0
Trema orientalis	109.7 ± 13.0	0
Macaranga tanarius	2.8 ± 0.7	0.3
Morinda citrifolia	27.6 ± 11.5	0
Bulbostylis barbata	10.2 ± 2.8	0.8
Fimbristylis dichotoma	1.9 ± 0.8	0
Ficus sp.	67.6 ± 36.3[b]	–
Pteris vittata	29.3[b, c]	–
Onychium siliculosum	5.3[b, c]	–

[a] $_{(\bar{x})}$ ± s.e., based on 4 replicates

[b] based on dry weight.

[c] based on a single determination.

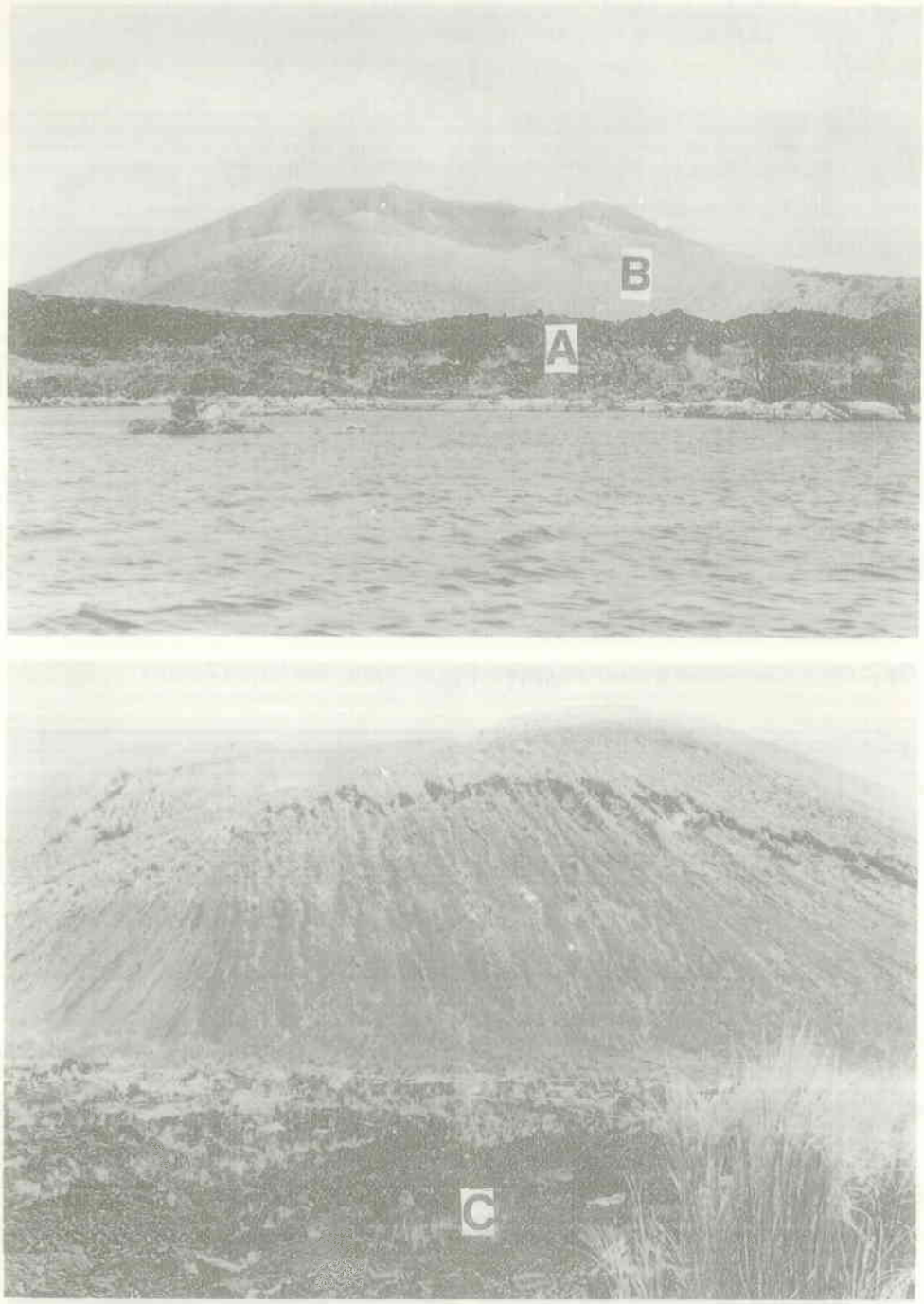

Fig. 1. Distant view of study area in Taal volcano showing (a) the area covered by huge volcanic rocks at the edge of the lake (A), the crest covered by deep deposit of loose volcanic ash (B); and (b) the crater floor covered with volcanic cinders (C).

Fig. 2. Photo of Saccharum spontaneum *showing long roots extending beyond 2 meters.*

Fig. 3. Well nodulated leguminous species, (a) Canavalia microcarpa *and (b)* Albizia procera.

NITROGEN FIXATION (ACETYLENE REDUCTION) IN THE ROOTS OF *BULBOSTYLIS BARBATA* (ROTTB.) KUNTH

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ABSTRACT

Bulbostylis barbata (Rottb.) Kunth, a pioneer sedge belonging to the family Cyperaceae, showed nitrogen fixation (acetylene reduction) in its roots. Isolation of the diazotrophs from its roots was done using the serial dilution technique followed by subculturing tube cultures with high nitrogenase activity on a semi-solid glucose yeast extract medium (SSGYE). Cultural, morphological and biochemical tests conducted on the isolates revealed that they belong to the family Enterobacteriaceae. *In vitro* acetylene reduction assay of the isolates in SSGYE gave positive nitrogenase activity.

INTRODUCTION

Bulbostylis barbata (Rottb.) Kunth, a sedge belonging to the family Cyperaceae was observed to be growing abundantly on the sandy slopes of Taal volcano (Aspiras *et al.*, 1985). Samples brought to the laboratory confirmed the suspicion that nitrogen fixation (acetylene reduction) was taking place in its roots. This prompted us to isolate and elucidate on the different characteristics of the diazotrophs involved.

Some workers have already reported the ability of non-nodulated plants to utilize atmospheric nitrogen through non-symbiotic association with certain species of bacteria. Most of these studies are centered on grasses but only recently Aspiras *et al* (1985) showed that there are many plant species other than grasses that harbor microorganisms capable of nitrogen fixation. The free-living nitrogen-fixing bacteria are found in at least 25 genera scattered in many different taxonomic groups. Line and Loutit (1971), reported nitrogen fixation of several bacteria isolated from tussock-grassland soil viz., *Clostridium butyricum, Bacillus circulans, B. polymyxa, Enterobacter aerogenes, Klebsiella pneumoniae* and *Escherichia intermedia.* An asymbiotic nitrogen fixation was also reported to occur in *Cyperus rotundus,* a sedge belonging to the family Cyperaceae with *Beijerinckia* sp. as the free-living nitrogen fixer (Ruschel and Britto, 1966). *Enterobacter cloacae* was observed to fix atmospheric nitrogen in loose association with the roots of corn

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(Raju *et al,* 1972), while several species of *Azospirillum* were reported to fix nitrogen in loose association with grasses (Dobereiner *et al,* 1972: Dommergues *et al,* 1973; Tjepkema, 1975).

This paper reports on the isolation and characterization of the nitrogen-fixing bacterial isolates found in the roots of *B. barbata.*

MATERIALS AND METHODS

B. barbata plants were collected from Taal volcano island and brought back immediately to the laboratory. Adhering soil particles were removed from the roots by washing them gently under tap water. Individual plants were placed in 50 mL erlenmeyer flasks and were assayed for nitrogenase activity using a Varian gas chromatograph. Roots of plants showing high nitrogenase activity were pooled together for isolation work. They were cut into 1-2 cm segments and approximately 1 g portions were placed in 200 mL erlenmeyer flasks containing 100 mL sterile distilled water. In an identical set, 5 g of glassbeads was added to effect mild maceration of roots upon shaking. Both flasks were shaken vigorously in a rotary shaker for 30 minutes. The bacteria dislodged by shaking without glassbeads were considered "outer root" bacteria or rhizosphere bacteria. Those obtained with glassbeads were considered "outer and inner root" bacteria (Barraquio and Watanabe, 1979). A series of ten-fold dilutions was prepared for both sets. Each dilution was inoculated to tubes containing 2 mL of semi-solid glucose yeast extract medium. One week later, the tubes were assayed for nitrogenase activity. From the tube cultures showing high nitrogenase activity, subcultures were made four more times at weekly intervals in order to enrich the bacteria and to facilitate the isolation of the diazotrophs. The last subculture was streaked onto plates with the same medium containing 1.5% agar. After 24 hours, only one type of bacterial colony appeared on all plates from all dilutions. Cultures were picked from single colonies and then further purified. Pure culture isolates were employed in *in vitro* acetylene reduction assay on semi-solid glucose yeast extract medium. Later, the isolates were subjected to different morphological, cultural and biochemical tests to facilitate their identification.

RESULTS AND DISCUSSION

Evidence of nitrogenase activity in the roots of *B. barbata* is presented in Table 1. Highest values were obtained in the 10^{-1} and 10^{-2} dilutions with the "outer + inner" root preparations giving higher values than the "outer root" preparation indicating the presence of the diazotrophs both in the "outer" and "outer and inner" portions of the roots. The effective inocula were observed up to the 10^{-2} dilution suggesting the rather low total population of the nitrogen fixers. The different isolates when subjected to nitrogenase assay (acetylene reduction) gave values ranging from 0.36 to 12.8 nmoles C_2H_4/tube/day (Table 2). However, large disparity in values obtained was attributed to the differences in the size of the inocula used.

The cultural and morphological characters of the seven isolates were identical. The colonies on nutrient agar after 24-48 hours appeared smooth, white to cream in

color, raised with entire end, round and slimy. Microscopic examination revealed that the cells were very short rods that appeared singly or in pairs, gram negative, non-spore former, and motile by peritrichous flagellation. Likewise, the biochemical tests conducted on all seven isolates yielded similar results (Table 3) suggesting the similar identity of the isolates.

Since only a limited number of characters were considered for purposes of identification, a definite taxonomic position of the isolates cannot be fully ascertained. However, their ability to produce acid and gas on SSGYE led us to believe that they belong to the family Enterobacteriaceae. A member of this family, *Enterobacter cloacae,* was reported to be responsible for nitrogenase activity in the roots of corn (Raju *et al,* 1972), and *Saccharum spontaneum* (Barraquio and Watanabe, 1979), and was likewise isolated from paper mill process waters (Neilson and Sparell, 1976), and from the intestines of man and animals (Bargesen and Hipsley, 1970).

SUMMARY AND CONCLUSIONS

Non-symbiotic nitrogen fixation was found to occur in the roots of *B. barbata* (Rootb.) Kunth, a pioneer sedge that thrives well in the sandy slopes of Taal volcano. The bacterium responsible for nitrogenase activity was isolated using the serial dilution technique after subculturing on a semi-solid glucose yeast extract medium (SSGYE). Results of the various cultural, morphological and biochemical tests showed that isolates from the "outer roots" and "outer and inner roots" were identical. Because of the ability to produce acid and gas in SSGYE, it was concluded that the organisms belong to the family Enterobacteriaceae. The free-living diazotrophs along with others that may be present in plant species found in the volcano island may hold the key to the vegetational development of this and other identically situated areas.

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Table 1. Acetylene reducing activity of the "outer root" and the "outer and inner root" bacteria from *B. barbata* grown in semi-solid glucose yeast extract medium (SSGYE) at various dilutions.

Source	Dilution	nmoles C_2H_4/tube/day[a]
"outer root"	10^{-1}	36.71
	10^{-2}	16.25
	10^{-3}	0.07
	10^{-4}	0.12
	10^{-5}	0.00
"outer and inner root"	10^{-1}	74.03
	10^{-2}	19.63
	10^{-3}	0.00
	10^{-4}	0.24
	10^{-5}	0.00

[a]average of three replicates per dilution.

Table 2. Acetylene reducing activity of pure isolates from *B. barbata* after two days of inoculation.

Isolate	Source	nmoles C_2H_4/tube/day[a]
Bb – 1	inner and outer root	0.36
Bb – 2	inner and outer root	0.75
Bb – 3	inner and outer root	1.24
Bb – 4	outer root	1.70
Bb – 5	inner and outer root	12.80
Bb – 6	inner and outer root	4.90
Bb – 7	inner and outer root	9.10

[a]average of three replicates per isolate.

Table 3. Biochemical properties of the bacterial isolates from *B. barbata*.

Biochemical test	Results/reactions
Growth on thioglycollate agar	facultative anaerobe
Catalase	+
Oxidase	–
Reaction to litmus milk	acidic
Voges-Proskaver	–
Methyl red	–
Citrate utilization	+
Nitrate reduction	–
phenylalanine deamination	–
indole production	–
starch hydrolysis	+
gelatin liquefaction	–
ACID FORM:	
glucose	+
sucrose	+
lactose	+
arabinose	+
raffinose	+
rhamnose	+
galactose	+
inositol	+
mannitol	+
xylose	+

THE UTILIZATION OF COCONUT WATER AS GROWTH MEDIUM FOR RHIZOBIA*

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ABSTRACT

Coconut water (CW) was obtained from 9-10-month-old coconuts and used as growth medium for rhizobial isolates from *Centrosema pubescens* Benth (centrosema), C_{11}, C_4; *Leucaena leucocephala* (Lam.) De Wit (ipil-ipil), L_{15}, L_5; *Vigna radiata* (L.) Wilczek (mungbean) M_5, M_4: *Arachis hypogaea* L. (peanut), P_3, P_7; and *Glycine max* (L.) Merr. (soybean), S_{38}, S_{13}. Growth responses of these isolates were observed after 4 days incubation of 30°C in the following media: aseptically collected CW (aseptic CW), pH 5.4; aseptic CW, pH adjusted to 7.0; sterilized CW, pH 5.4; sterilized CW, pH 7.0; sterilized CW + NH_4Cl (50 mg/100 mL), pH 5.2; sterilized CW + K_2HPO_4 (100 mg/100 mL), pH 6.0; yeast extract mannitol broth (YEMB), pH 7.0; and basal medium (BM), pH 7.0.

All rhizobial isolates grown in CW or CW-based media showed good growth except C_4 and M_5 which had lower growth response in sterilized CW and lower pH. There was also higher growth response and more viable cells of CB81, L_{15} and M_5 in CW than in YEMB or BM. Inoculum from rhizobia grown in CW can infect their host plants although they showed varying degrees of nitrogen-fixing activity.

INTRODUCTION

The most commonly used culture medium for growing *Rhizobium* is yeast extract mannitol broth (YEMB) or yeast extract mannitol agar (YEMA). These consist mainly of yeast extract, mannitol, glutamate and mineral salts ($MgSO_4$, $CaCO_3$, KH_2PO_4, NaCl, etc.). In comparison, coconut water has a much richer composition. It contains not only the necessary carbon substrates for energy and growth but also the important amino acids, a combination of several sugars, sugar alcohols, organic acids, vitamins, and growth factors. It has been reported that 17 species of bacteria and 8 species of yeast could be grown in coconut water medium (Alejar, 1974). Combined with different kinds of agar it has also been found to support growth of 22 bacterial and yeast species (Gonzales, 1975).

This study was conducted to see if coconut water can also be utilized as a culture medium for rhizobia.

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MATERIALS AND METHODS

Coconut water obtained from 9-10-month-old coconuts was utilized in the preparation of 6 types of growth media. These were: 1) aseptic CW, pH 5.4; 2) aseptic CW, pH adjusted to 7.0; 3) CW (obtained without precautions to insure aseptic conditions), pH 5.4; 4) CW, pH adjusted to 7.0; 5) CW + NH_4Cl (50 mg/100 mL), pH 5.2; 6) CW + K_2HPO_4 (100 mg/ 100mL), pH 6.0; 7) YEMB consisting in g/L of mannitol, 10; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; yeast extract, 1.0; and $CaCO_3$, 0.1 or saturation, pH 7.0; 8) Basal medium (BM) (Elkan and Kwik, 1968) consisting in g/L of K_2HPO_4, 1.0; KH_2PO_4, 1.0; NaCl, 0.2; $MgSO_4$, 0.18; $FeCl_3$, 0.04; NH_4Cl, 0.5; glutamate, 0.2; mannitol, 5.0; and $CaSO_4$, 0.1 (saturation), to exclude any undissolved $CaSO_4$ which sometimes one encounters when using 0.1 g/L $CaSO_4$. The pH of the medium was measured by an Orion 301 pH meter and adjusted to 6.8-7.0.

Aseptic collection of coconut water was done by first spraying the soft area of the nut (eye) with alcohol followed by flame sterilization. A small opening was made with a sterile knife and immediately plugged with a sterile wad of cotton. For the growth experiments, 5 mL of aseptic CW was drawn out with a sterile syringe and introduced into sterile 25 mL test tubes plugged with sterile wads of cotton.

Five mL of each culture medium except media 1 and 2 was placed in 25-mL test tubes, plugged with cotton wads and steam sterilized at 120°C at 15 psi for 15 minutes.

The inoculum was prepared from 10 rhizobial isolates: C_4, C_{11} from *Centrosema pubescens* (centrosema); L_5, L_{15} from *Leucaena leucocephala* (ipil-ipil); M_4, M_5 from *Vigna radiata* (mungbean); P_3, P_7 from *Arachis hypogaea* (peanut) and S_{13}, S_{38} from *Glycine max* (soybean). The isolates were cultured in YEMB for a week, harvested, and washed in physiological saline solution and optical density was adjusted to 0.2.

Each of the media was inoculated with 0.1 mL inoculum and incubated at 30°C in a circulating water bath for 4 days. Optical density measurements at 420 nm were taken after incubation using a Bausch and Lomb Spectronic spectrophotometer. Determinations were done in duplicate for each isolate and duplicate controls (blanks) for each type of medium were included. Growth responses were rated as follows: $O.D._{420nm}$ 1.0 or greater = 4; 0.7 to 0.9 = 3; 0.4 to 0.6 = 2; 0.3 to 0.1 = 1; and reading less than 0.1 = slight growth.

A set of experiments was conducted to determine dry matter yield or cell biomass and plate count on yeast extract mannitol agar (YEMA) of ipil-ipil isolates, L_{15} (native strain) and CB81 (Australian strain), and mungbean isolate M_5 grown in CW and YEMB. Duplicate flasks containing CW and another duplicate set containing YEMB were inoculated with 1-week old agar slant cultures (1 slant per flask). Uninoculated flasks for each medium were included as controls. Flasks were incubated under shaken conditions for 3-5 days for fast growers and 7-10 days for slow growers. For cell dry weight or cell yield determination, 10 mL of the above broth was filtered through a Millipore at 15 psi and

provided with a 0.45 μm pore size filter paper. Ten mL of uninoculated media was also included and filtered as controls. Each filter paper was placed in a Petri dish and dried at 60°C in an oven overnight. After drying, dry weights of the samples were taken in an analytical balance.

Plate count of viable rhizobial cells was taken according to the procedure of Vincent (1970). Cells were harvested by centrifugation. One mL of each dilution was plated in 15 mL of melted and cooled YEMA. After thorough mixing, the agar was then allowed to solidify, and the dish was incubated at 28°C in an inverted position for 3 days for fast growers and 7-10 days for slow growers.

For nodulation studies, L_{15}, CB81, and M_5 grown in CW, CW + NH_4Cl and YEMB were used to inoculate their host plants. The seeds were first surface-sterilized for 2-3 min with 95% alcohol, then with 10% chlorox for 3-5 min followed by 4-5 rinses of sterile water. The sterile seeds were then allowed to germinate in sterile filter paper in polypropylene growth pouches containing seedling solution (Specht *et al*, 1956) as shown in Figure 1. In the case of ipil-ipil the seeds were first dipped in 80°-85°C water for 10 min before sterilizing. The growth pouches were held upright between supports of a record rack. These were then placed in a screened growth chamber illuminated by two 40-watt fluorescent lamps (∿12,000 lux). The temperature of the chamber was set at 25°C ± 2°C.

Three days after germination, the seedlings were inoculated with the rhizobial broth cultures. Any loss of liquid nutrient solution was replaced periodically during the period of growth of the seedlings. The seedlings were taken out of the growth pouches after 6 weeks of growth and decapitated from their roots. The roots with the nodules were then assayed for nitrogenase activity.

The nitrogenase activity of the nodules was determined by the acetylene reduction method (Hardy *et al*, 1973). The roots with the nodules were placed in the barrel of a 25-mL syringe and the plunger pushed to expel air and at the same time, to pack the sample to fill a volume of 5 mL. The tip on the other end of the barrel was then sealed with a rubber silicone septum. Two mL acetylene and 18 mL of air were injected into the incubation chamber as shown in Figure 2. The gases inside the incubation chamber were mixed thoroughly by shaking the syringe vigorously up and down and side to side for about a minute. The sample was allowed to incubate for 2 hours. Gas samples (0.1 to 0.5 mL) were withdrawn after the incubation period and injected into a gas chromatrograph (Varian Aerograph, series 1400). Instrument settings are: column temperature, 55°C; injector temperature, 45°C; detector temperature, 100°C; range, 10^{-12} amps/mV; attenuation, 32.

RESULTS AND DISCUSSION

The results of culturing 10 tropical rhizobial isolates in different types of coconut water media (Table 1) indicate that aseptic CW and steam-sterilized CW, with or without

pH adjustments to 7.0 are good growth media for the strains tested. Addition of ammonium chloride reduced the pH of the coconut water by 0.2 pH unit but this did not affect the growth of the rhizobia. It must be noted that there is an increase of about 0.3 optical density unit for this medium over that of aseptic CW. Addition of potassium phosphate salts did not affect growth appreciably in comparison with aseptic CW.

Growth responses of C_4 and M_5 were lower in sterilized CW but adjustment of pH of medium to 7.0 improved the growth response of M_5. A slight depression of growth was observed for P_7 in sterilized CW, pH 7.0.

The lower growth responses of M_5 in sterilized CW as compared to that in aseptic CW might be due to changes in buffering capacity of CW and destruction during sterilization of heat-labile compounds essential for growth of M_5. C_4 and M_5 strains were quite sensitive to pH as shown by their lower growth in sterilized CW, pH 5.4 and improved growth in sterilized CW, pH 7.0.

The good growth of the rhizobial isolates in aseptic coconut water as well as in the other coconut-based media may be attributed to the rich and complex composition of coconut water and the right proportion of these components to each other to provide a good growth medium not only for plant cells but also for rhizobia. Raghavan (1976) made a compilation of the nutrients, organic acids, growth hormones and miscellaneous substances of coconut milk (term for coconut water by some researchers). Sugars (Tulecke *et al,* 1961) and alcohol sugars (Dunstan, 1906; Pollard *et al,* 1961) present in mg/mL are: sucrose, 9.18; glucose 7.25; fructose, 5.25; sorbitol, 15; *myo*-inositol, 0.1; *scyllo*-inositol, 0.5 and mannitol, 0.08. Organic acids (Tulecke *et al,* 1961) present in meq/mL are: malic acid, 0.3431; shikimic acid and other acids of similar structures, 0.57; citric acid, 0.37; and pyrrolidine carboxylic acid, 0.39. The inorganic ions (mg/100 g) in coconut water (McCance and Widdowson, 1940) are: potassium, 312; chlorine, 183; sodium, 105; phosphorus, 37; magnesium, 30; sulfur, 24; iron, 0.10; and copper, 0.04.

Amino acids present in coconut water (Tulecke *et al,* 1961; Steward *et al,* 1961) in μg/mL are: aspartic acid, 65; threonine, 44; serine, 111; asparagine and glutamine, 60; proline, 97; glutamic-acid, 240; alanine, 312; valine, 27; methionine, 8; isoleucine, 18; leucine, 22; tyrosine, 16, β-alanine, 12; γ-aminobutyric acid, 820; lysine, 39; trytophan, 39; arginine, 133; ornithine, 22; histidine, trace; pipecolic acid, +; hydroxyproline, trace; phenylalanine, 12; glycine, 13.9; homoserine, 5.2; and cystine, 0.97 – 1.17 g/100 protein. Vitamins (Vandenbelt, 1945) and growth hormones are also present. Vitamins in μg/mL are: nicotinic acid, 0.64; pantothenic acid, 0.52; biotin, 0.02; riboflavin, 0.01; folic acid, 0.003; and thiamine and pyridoxine in traces. Growth hormones are: auxins (Paris and Duhamet, 1953), 0.07 mg/mL; gibberelins (Radley and Dear, 1958), trace; and cytokinins (Zwar *et al,* 1963), trace. Other morphogenetic substances such as those which stimulate legume nodule are also present in CW (Valera and Alexander, 1965; Schaffer and Alexander, 1967).

A comparison of growth responses of the isolates to aseptic CW, sterilized CW, YEMB and BM are given in Table 2.

Higher growth responses are evident in CW media than in the traditional YEMB culture medium and the basal medium. Comparison of growth responses between YEMB and BM showed better growth of the isolates in YEMB. However, results of the 7-day incubation period of BM were comparable to that for YEMB having a 4-day incubation period. The higher growth rates in YEMB may be due to the presence of growth-stimulating factors such as amino acids, vitamins and hormones in yeast extract.

Cell dry weights or cell yields of CB81, an Australian ipil-ipil isolate and mungbean isolate, M_5 grown in CW did not show much difference in the weights obtained from those cultures in YEMB. However, L_{15}, the native effective ipil-ipil isolate has a higher cell yield in CW than in YEMB (Table 3).

The results in Table 4 are quite interesting. There are more viable cells of CB81 and L_{15} in CW than in YEMB. M_5, however, has a much lower viable cell count in CW than in YEMB. It should be noted though, that CW has a natural pH of 5.4 and that YEMB has a pH of 7.0. Results of growth experiments in test tubes of M_5 showed a similar reduction in sterilized CW (pH 5.4) over that of sterilized CW (pH 7.0). These observations seem to indicate that M_5 is more viable in a neutral medium.

Initiation of nodulation of the ipil-ipil seedlings was observed during the third week of growth. The ipil-ipil seedlings inoculated with CB81 grown in YEMB did not yield nodules during this growth period.

The nitrogenase activity of the nodules of ipil-ipil and mungbean seedlings gives a measure of their nitrogen fixing ability. In most cases, a factor of 3 is used to convert ethylene produced by the reduction of acetylene to nitrogen fixed. Under the conditions of the experiment, it was noted that nodules produced by ipil-ipil inoculated with L_{15} had a higher nitrogenase activity than those obtained from plants inoculated with CB81 whether grown in CW or YEMB. As shown in Table 5, nodules from mungbean inoculated with M_5 grown in CW + NH_4Cl have lower nitrogenase activity than those nodules from plants inoculated with M_5 grown in CW and YEMB. The nitrogenase activity of nodules from plants inoculated with M_5 grown in CW + NH_4Cl may have been depressed by the low pH and the availability of ammonium nitrogen in the medium.

SUMMARY AND CONCLUSIONS

Rhizobial isolates from *C. pubescens* Benth (centrosema) C_4, C_{11}; *L. leucocephala* (Lam.) De Wit, L_5, L_{15}; *V. radiata* (L.) Wilczek (mungbean), M_4, M_5; *A. hypogaea* L. (peanut), P_3, P_7; and *G. max* (L.) Merr. (soybean), S_{13}, S_{38} were grown in coconut water (CW) from matured coconuts (9-10 months old). 6 types of CW media, yeast extract mannitol broth (YEMB) and basal medium (BM) were used as culture media.

All rhizobial isolates grew well in aseptic CW and sterilized CW after 4 days at 30°C. However, C_4 and M_5 grew better in sterilized CW at pH 7.0 than at pH 5.4. Growth responses in YEMB and BM are relatively lower than in CW media. Cell dry weight of CB81 (Australian ipil-ipil isolate) and L_{15} (Native ipil-ipil isolate) are higher in CW than in

YEMB. Plate count of viable rhizobia of CB81 and L_{15} are also higher in CW. However, plate count of M_5 is higher in YEMB than in CW (3.0×10^8 CW; 5.5×10^9 YEMB).

Nodules produced by ipil-ipil inoculated with L_{15} had higher nitrogenase activity than those inoculated with CB81. Whether grown in CW or YEMB. Nodules from mungbean inoculated with M_5 grown in CW + NH_4Cl have lower nitrogenase activity than those nodules from plants inoculated with M_5 grown in CW and YEMB.

The following conclusions may be drawn from the overall experimental results: 1) CW, either aseptically collected or sterilized can be used as growth medium for *Rhizobium,* 2) the rhizobial isolates tested tend to grow faster in CW than in YEMB, 3) inoculum from *Rhizobium* cultured in CW can effectively infect their host plants and form nodules that can fix nitrogen.

The results from these studies await further verification under greenhouse and field conditions. If indeed the results would show that the rhizobia grown in coconut water would be effective in nodulating their respective hosts, then preparations should be made to mass produce inocula using coconut water as the basic medium. The cost of inoculant mass production would be lower than the one using the standard culture medium since coconut water is readily available in large quantities. The cost of CW will come from handling and transportation from dessicated coconut factories. A lot of savings will also be realized if coconut water instead of YEMB will be utilized since most of the ingredients that make up YEMB are imported. Research work on the applicability of adding coconut water-based liquid inoculant directly into the soil where the seeds are sown should be conducted and compared with the traditional methods of applying mass inoculants.

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Table 1. Growth responses of 10 tropical rhizobial isolates to 6 types of growth media utilizing CW.

Rhizobial Isolate	Growth Medium, CW					
	Aseptic pH 5.4	Aseptic pH 7.0	Sterilized pH 5.4	Sterilized pH 7.0	+NH_4Cl[a] pH 5.2	+K_2HPO_4[b] pH 6.0
	O.D.$_{420\ nm}$ and (Score)					
C_4	1.4 (4)	1.4 (4)	0.7 (3)	0.8 (3)	1.1 (4)	1.0 (4)
C_{11}*	1.4 (4)	1.3 (4)	1.5 (4)	1.4 (4)	2.0 (4)	1.2 (4)
L_5[c]	1.4 (4)	1.5 (4)	1.6 (4)	1.2 (4)	1.9 (4)	1.2 (4)
L_{15}*, [c]	1.5 (4)	1.6 (4)	1.8 (4)	1.5 (4)	2.0 (4)	1.8 (4)
M_4	1.3 (4)	1.3 (4)	1.3 (4)	1.1 (4)	1.5 (4)	1.5 (4)
M_5*	1.4 (4)	1.2 (4)	0.8 (3)	1.1 (4)	1.5 (4)	1.2 (4)
P_3*	1.4 (4)	1.3 (4)	1.4 (4)	1.4 (4)	1.7 (4)	1.5 (4)
P_7	1.3 (4)	1.1 (4)	1.1 (4)	0.9 (3)	1.4 (4)	1.1 (4)
S_{13}	1.4 (4)	1.5 (4)	1.5 (4)	1.2 (4)	2.0 (4)	1.4 (4)
S_{38}*	1.4 (4)	1.4 (4)	1.3 (4)	1.2 (4)	1.7 (4)	1.1 (4)

[a]50 mg/100 mL.

[b]100 mg/100 mL.

[c]L_5 and L_{15} exhibits gummy growth.
*More effective strain.

Table 2. Growth responses of 10 tropical rhizobial isolates to CW, YEMB and BM.

Rhizobial Isolate	Growth Medium				
	Aseptic CW pH 5.4	Sterilized CW pH 5.4	YEMB pH 7.0	BM pH 7.0	BM[a] pH 7.0
	O.D.$_{420\ nm}$ and (Score)				
C_4	1.4 (4)	0.7 (3)	0.5 (2)	0.2 (1)	1.3 (4)
C_{11}	1.4 (4)	1.5 (4)	0.7 (3)	0.2 (1)	1.4 (4)
L_5[b]	1.4 (4)	1.6 (4)	0.8 (4)	0.3 (4)	1.6 (4)
L_{15}*, [b]	1.5 (4)	1.8 (4)	0.8 (4)	0.3 (4)	1.8 (4)
M_4	1.3 (4)	1.3 (4)	0.7 (3)	0.2 (1)	1.2 (4)
M_5*	1.4 (4)	0.8 (3)	0.5 (2)	0.2 (1)	0.3 (2)
P_3*	1.4 (4)	1.4 (4)	0.5 (2)	0.2 (1)	0.4 (2)
P_7	1.3 (4)	1.1 (4)	1.1 (4)	1.0 (4)	1.4 (4)
S_{13}	1.4 (4)	1.5 (4)	0.8 (3)	0.8 (3)	1.3 (4)
S_{38}*	1.4 (4)	1.3 (4)	0.8 (3)	0.2 (1)	0.7 (3)

[a] 7-day incubation period.

[b] L_5 and L_{15} formed clumps of gummy growth; given a score of 4 for the presence of dense clumps even if O.D. lower than 1.
*More effective strain.

Table 3. The cell dry weight or cell yield of L_{15}, CB81 and M_5 grown in CW and in YEMB.

Growth medium	Ipil-ipil isolate CB81	Ipil-ipil isolate L_{15}	Mungbean isolate M_5
	Cell Dry Weight, mg/10 mL		
CW	141.8	150.3	2.4
YEMB	141.0	124.0	2.5

Table 4. Plate count of rhizobia on CW and on YEMA.

Growth medium	Ipil-ipil isolates CB81	Ipil-ipil isolates L_{15}	Mungbean isolate M_5
	Number of cells/mL		
CW	5.5×10^7	38.5×10^7	3.0×10^8
YEMA	2.0×10^7	14.0×10^7	5.5×10^9

Table 5. The nitrogenase activity of nodules produced by ipil-ipil isolates CB81, L_{15} and mungbean isolate, M_5 cultured in CW, CW + NH_4Cl, and YEMB.

Growth medium	Ipil-ipil isolates CB81	Ipil-ipil isolates L_{15}	Mungbean isolate M_5
	Nitrogenase Activity (nanomoles C_2H_4 produced/mg fresh wt. nodule/min)		
CW	0.01	0.04	0.04
CW + NH_4Cl	0.01	0.04	0.01
YEMB–	–	0.03	0.03

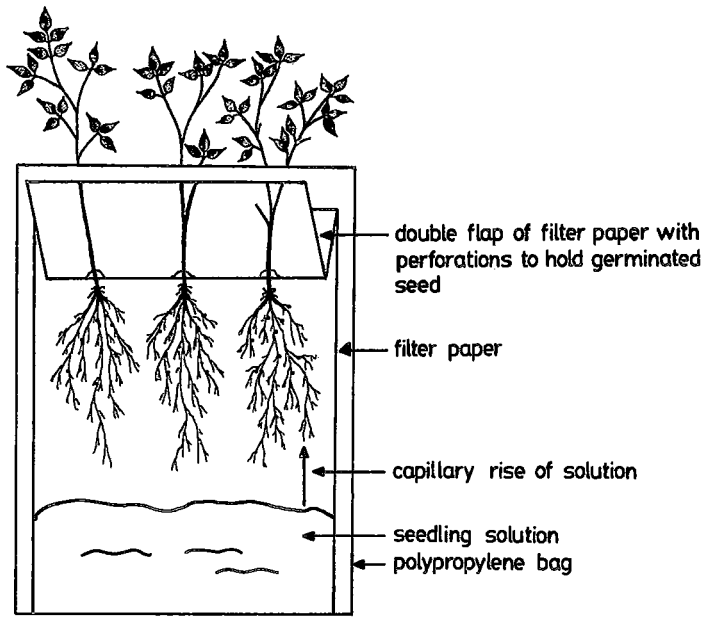

Fig. 1 Diagram of a filter paper polypropylene growth pouch for growing legume seedlings inoculated with Rhizobium *strains grown in coconut water and YEMB.*

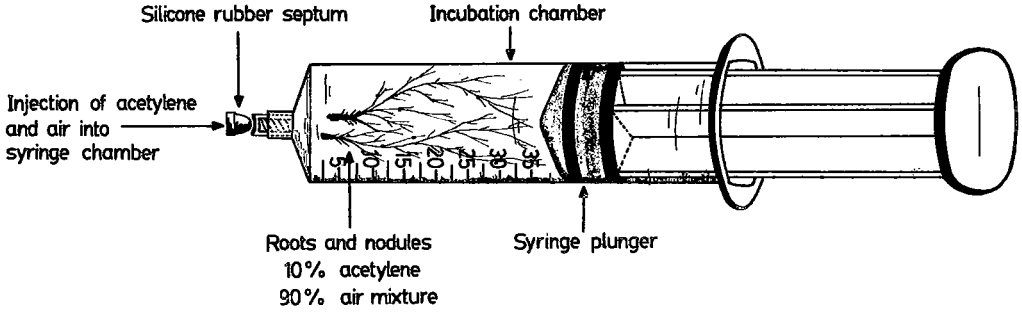

Fig. 2. Diagram of incubation chamber for the acetylene-ethylene assay utilizing the barrel of a disposable syringe.

LIGHT AND PHOSPHORUS INTERACTION ON GROWTH, NITROGEN FIXATION AND MINERALIZATION OF *AZOLLA PINNATA*

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ABSTRACT

Increasing light intensity increased biomass production, reduced phosphorus uptake, decreased heterocyst frequency at high levels of phosphorus and increased carbon content of tissues. It did not, however, affect nitrogen content. Maximum mineralization of nitrogen occurred between 9-12 weeks and was delayed in the light-exposed system compared to those in darkness.

INTRODUCTION

The symbiotic nitrogen fixing ability of the *Azolla-Anabaena* association makes it a good source of organic nitrogen. Hence the cultivation of *Azolla* is gaining acceptance in local irrigated rice farming. However, scattered observations and feedback information from farmers indicate that the growth pattern of specific *Azolla* species vary in different geographical areas. This clearly illlustrates the likewise reported environmental factors and interactions which limit the population growth and subsequent utilization of the aquatic fern (Lumpkin and Plucknett, 1980; Moore, 1969; Talley and Rains, 1980).

Light intensity and phosphorus (P) availability are two of the many factors which limit the growth of *Azolla*. According to the studies by Ashton (1974) and Talley and Rains (1980), the growth of *Azolla* saturates at approximately 25 to 50% of full sunlight or approximately 25 to 50 klux. However, most species exposed to such light irradiance develop anthocyanin pigment (Moore, 1969; Olsen, 1972; Holst, 1977) which is also a manifestation of P deficiency resulting in reductions in biomass, nitrogen content and acetylene reduction activity (Watanabe *et al*, 1977; Subudhi and Watanabe, 1979).

This paper reports on the initial investigation on the physiology of *Azolla* particularly the effect of light intensity and P levels and their interactions on growth, phos-

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phorus, nitrogen and carbon content of the fern and heterocyst formation of the *Anabaena* symbiont. The effect of light on the mineralization of *A. pinnata*, a species indigenous to the Philippines, (Sweet and Hills, 1971) was also presented.

MATERIALS AND METHODS

Batch cultures of *Azolla* weighing 55 mg (7.9 g m^{-2}) were grown in nitrogen-free culture medium contained in glass beakers with varying levels of P such as 0, 40 and 80 ppm. The cultures were incubated under controlled conditions with light intensities of 2.5, 12 and 52 klux. Samples of plants were taken after 7 days of treatment and analyzed for total nitrogen (Yoshida *et al,* 1972), phosphorus (PCARR, 1980) and carbon (Black *et al,* 1965). The number of heterocysts were counted from gently macerated leaflets using a light microscope.

Mineralization of *A. pinnata* was measured by incorporating fresh *Azolla* into known weights of soil to give an equivalent rate of 30 kg N/ha. The samples were kept in sealed bottles under dark or light (5 klux with 12 h photoperiod) conditions. Weekly determinations for NH_4-N were taken following the procedures outlined by Bremner and Keeney (1966).

All the data were subjected to statistical analysis using the completely randomized design.

RESULTS AND DISCUSSION

Effect on biomass production

The main effect of light on the symbiotic relationship between the host *Azolla* and the symbiont *Anabaena azollae* is on the ability of the former to harness light energy for photosynthesis and the latter to utilize light energy for the nitrogen fixation process (Peters *et al,* 1980). Under favorable conditions, the fern-algal association can double in fresh weight every 3-5 days depending on the species (Watanabe *et al,* 1977). Figure 1 shows that the fresh weight of *A. pinnata* increased as light intensity increased at all P levels. The highest biomass production was obtained at 52 klux which gave a 306% increase over that obtained at 2.5 klux at 40 ppm P. It has been reported, however that optimum growth in *A. pinnata* was obtained at a lower light intensity of 25 klux (Lumpkin and Plucknett, 1980; Lu *et al,* 1963).

When ferns were exposed to a light intensity of 12 klux, growth was not increased by application of P concentrations greater than 5 ppm (Watanabe *et al,* 1980) which is in contrast to our findings that higher P level in the medium enhances biomass production. For example, at 80 ppm P, growth of the fern at the same light intensity was significantly increased by 49% (Fig. 1).

Effect on phosphorus and nitrogen uptake

Increasing light intensity from 12 klux to 52 klux decreased the phosphorus uptake of *A. pinnata* (Fig. 1). This differential uptake of phosphorus by the fern in response to

varying intensities of light suggest that light can influence the capacity of *Azolla* to absorb P from the medium. Values for P content reported in this experiment range from 0.4% to 1.4% (dry weight basis) which is comparable to the reported 0.6 – 1.5% range (Anonymous, 1975). These values are high but luxury consumption of P can be exhibited by various species of *Azolla* (Lumpkin and Plucknett, 1981).

When *A. pinnata* was exposed to 50% full sunlight or about 52 klux, there was a significant reduction in the P content of the tissues at 40 and 80 ppm compared to that in the absence of the element. It is apparent from the results that P uptake is proportionate to the amount of added P at the same light intensity.

The results showed that light did not affect the nitrogen content of tissues (Table 1). However, there are indications that increasing light intensity increased nitrogen content of the tissues at all phosphorus levels but results were not significant. For example, the 52 klux treatment resulted in higher total nitrogen content compared to tissues exposed to lower light intensities. Other workers reported high nitrogen content at 47 klux in the same species (Lumpkin and Plucknett, 1980; Lu *et al,* 1963). On the other hand, deficiency of P in the medium did not likewise alter nitrogen content (Table 1). This is in contrast with the reported reduction in nitrogen content with P deficiency using *A. pinnata* (Watanabe *et al,* 1977; Subudhi and Watanabe, 1979).

In addition to chlorophyll and carotenoids, most *Azolla* species produce a red or anthocyanin pigment in response to high light intensity (Moore, 1969; Olsen, 1972; Holst, 1977) or to a deficiency in phosphorus (Watanabe *et al,* 1980; Shi Ding-ji *et al,* 1981). These observations were not exhibited in the present *Azolla* cultures in agreement with reports that some species/strains can remain free of anthocyanin even when exposed to full sunlight (Talley and Rains, 1980; Watanabe *et al,* 1980). Samples of "red" *A. pinnata* collected from the propagation field and exposed to direct sunlight was found to contain 2.40% total nitrogen while the anthocyanin-free *Azolla* from the same field in a shaded area contained 3.95%.

Effect on heterocyst formation

The activity of the enzyme nitrogenase, which is believed to occur in the heterocyst of the symbiont, is largely dependent on light. It has been reported that decline in enzyme activity is more rapid when light intensity increased than when light intensity decreased (Ashton, 1974). About 10 klux was required for saturation in *A. imbricata* (Shi Ding-ji *et al,* 1981) and in another study it was noted that low light increased heterocyst frequency (Fogg, 1949). Since nitrogenase activity is affected by light intensity (Ashton, 1974; Shi Ding-ji *et al,* 1981) and the enzyme activity paralleled increase in heterocyst number (Haselkorn, 1978; Hill, 1977) data shown in Fig. 2 support the hypothesis that low light intensity can induce nitrogenase activity as shown by the high heterocyst frequency more marked in the presence of phosphorus. On the other hand, when the fronds were exposed to 52 klux, high heterocyst frequency is also obtained in the absence of phosphorus.

In view of the dependence of nitrogenase activity on the reductant from prior photosynthesis and ATP from cyclic photophosphorylation (Peters, 1977), it is suggested

that there should be available P or high light intensity to enhance the production of ATP and/or reductant which will consequently increase enzyme activity. This is indirectly demonstrated by the observation on high heterocyst frequency (Fig. 2) substantiated by the high nitrogen content of the tissues (Table 1). Determination of the nitrogenase activity can strengthen this claim but this was not determined in this study. The finding that P uptake was reduced at 52 klux treatment (Fig. 1) is likely to benefit formation of heterocyst since at high light intensity, low/absence of phosphorus promotes heterocyst formation (Fig. 2).

Mineralization of Azolla pinnata

Slow decomposition gives a slow release effect which is ideal for efficient absorption of released nutrients by the rice crop. The C:N ratio affects the amount and decomposition rate of soil-incorporated *Azolla* (Watanabe *et al,* 1977; Li Shi-ye, 1982; Shi *et al,* 1980; Tujimura, 1977) and because of the low C:N ratio reported for *Azolla* (Talley and Rains, 1980; Peters, 1977; Lumpkin *et al,* 1982; Peters *et al,* 1980), this aquatic fern is very effective as a green manure. It was pointed out that mineralization rate in paddy soils of Eastern China differs with the species. *A. imbricata* had a higher rate of mineralization than *A. filiculoides;* the former species having a lower C:N ratio than the latter (Shi *et al,* 1980). In laboratory-sterilized cultures, ammonia released in solution was much higher in *A. mexicana* compared to traces of ammoniacal nitrogen released by *A. filiculoides* (Talley and Rains, 1980). In the same experiment, it was also reported that low light diminished rate of ammonification. This is in contrast with our results on unsterilized cultures where increase in light intensity increased the carbon content of tissues resulting in a higher C:N ratio and a lower amount of mineralized nitrogen (Table 2).

Mineralization of *Azolla* under field and laboratory conditions (Watanabe *et al,* 1977; Li Shi-ye, 1982) took place during a wide range of 2-8 weeks after incorporation. Figure 3 shows mineralization of *A. pinnata* carried out in the laboratory under light and dark conditions at 30°C. Maximum ammonia released in the soil was attained 9-12 weeks after incorporation in both light and dark incubation. It seems that the activity of the microbial flora was enhanced by light during the second and the third weeks of incubation when 12% of ammoniacal nitrogen was released. However, the 21% maximum mineralization of *A. pinnata* occurred two weeks earlier in the dark than in the light. These observations, however, are of mainly theoretical interest since mineralization normally takes place in the dark.

SUMMARY AND CONCLUSIONS

The assessment of the interaction of the effect of light intensity and phosphorus levels was carried out using *A. pinnata* grown for 7 days in minus nitrogen culture solution. Results indicated that light can influence (1) biomass production (2) the capacity of *A. pinnata* to absorb phosphorus from the medium and (3) heterocyst formation but (4) appeared to have no effect on total nitrogen content of tissues.

At high light intensity (52 klux) P uptake is reduced with the addition of P whilst lower light intensity (12 klux) tends to increase P uptake with increasing concentration of

P. Heterocyst formation is enhanced either with high light intensity (52 klux) in the absence of P or low light (2.5 klux) in the presence of P. Therefore the reduction of P uptake at 52 klux benefited heterocyst formation. However, these findings were observed only in one strain of one species and therefore may not be true for other species/strain. Nevertheless this high light-induced or phosphorus-induced heterocyst formation will expectedly increase nitrogen fixation. Further assessment of nitrogenase activity under the same experimental conditions is however recommended.

ACKNOWLEDGEMENTS

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Table 1. The effect of light intensity on nitrogen and carbon content of *A. pinnata* grown in deficient nitrogen solution and three levels of phosphorus for 7 days. Values are means of 12 observations. Means of the same letter are not significantly different.

	0 ppm P		40 ppm P		80 ppm P	
Light intensity Klux	Nitrogen %	Carbon %	Nitrogen %	Carbon %	Nitrogen %	Carbon %
2.5	3.10 a	12.24 b	3.61 a	6.40 a	3.49 a	8.95 a
12.0	3.80 a	14.49 b	3.91 b	13.89 b	4.17 a	18.87 c
52.0	3.90 a	25.58 d	3.81 a	29.41 da	4.19 a	27.48 d

Table 2. The effect of light intensity on nitrogen and carbon content of *Azolla pinnata* grown in deficient nitrogen solution and three levels of phosphorus for 7 days. Inoculum was 55 mg (7.9 mg m^{-2}) values are means of 12 observations. Means of the same letter are not significantly different.

	0 ppm P		40 ppm P		80 ppm P	
Light intensity Klux	Nitrogen %	Carbon %	Nitrogen %	Carbon %	Nitrogen %	Carbon %
2.5	3.10 a	12.24 b	3.61 a	6.40 a	3.49 a	8.95 a
12.0	3.80 a	14.49 b	3.91 a	13.89 b	4.17 a	18.87 c
52.0	3.90 a	25.58 d	3.81 a	29.41 de	4.19 a	27.48 d

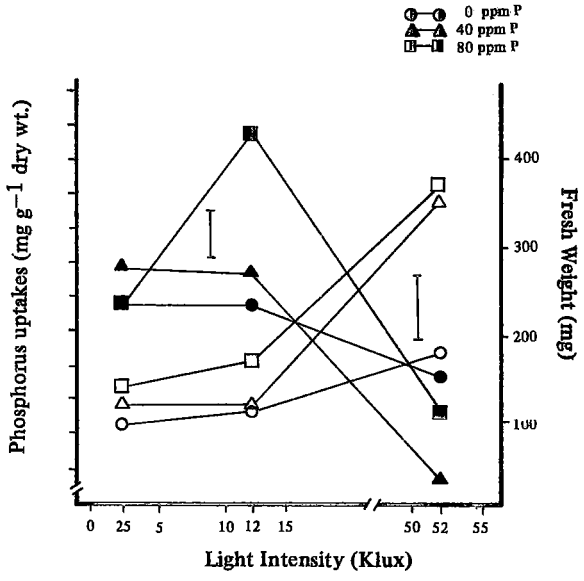

Fig. 1. The effect of light intensity on the fresh weight (open symbols) and phosphorus uptake (closed symbols) of **Azolla pinnata** *grown in 3 levels of phosphorus after 7 days. Values are means of 12 observations. Least significant difference (LSD.05) at 5% level of significance is shown as vertical bar.*

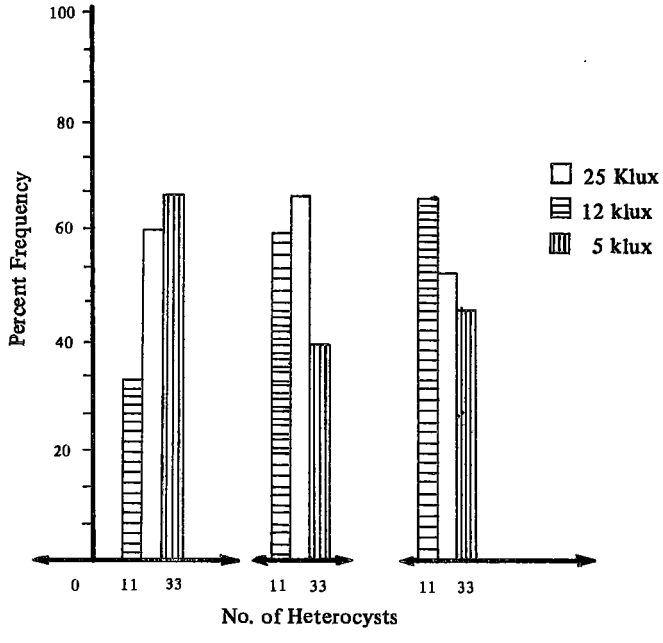

Fig. 2. The effect of light intensity and phosphorus level on the number of heterocysts of **Anabaena Azollae** *at class boundaries where it was observed to be at highest frequency. Values are means of 15 observations.*

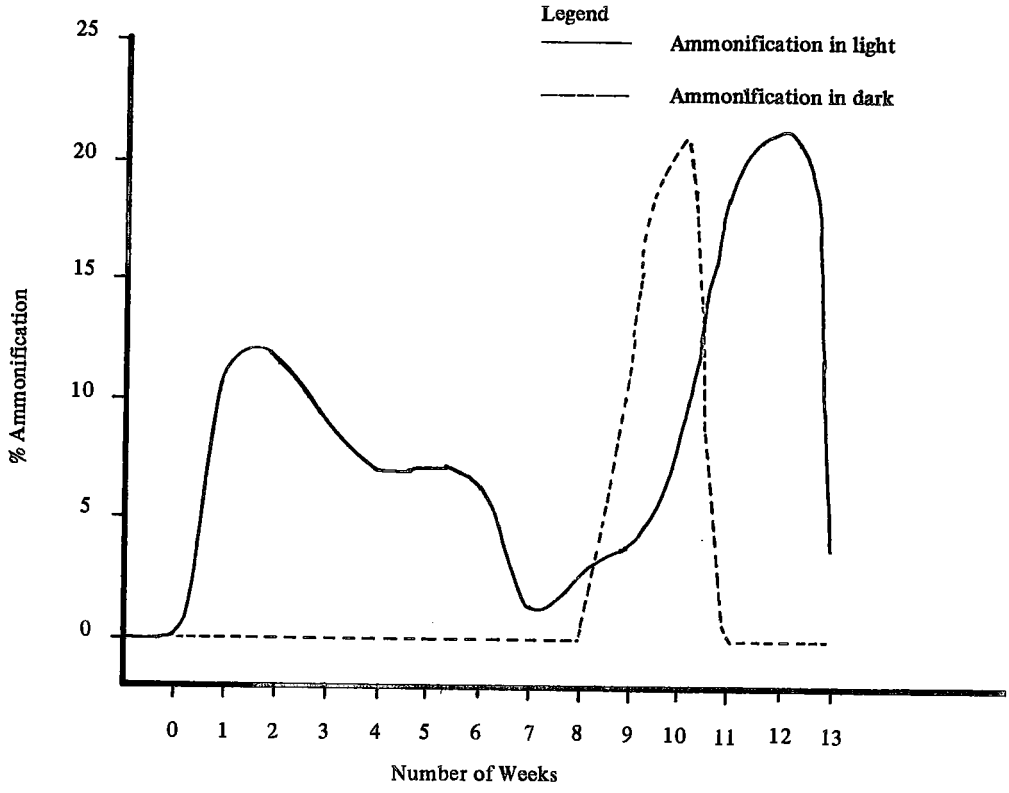

Fig. 3. Ammonification of A. pinnata *applied at a rate of 30 kg N/ha under dark and light conditions. Values are means of 2 observations.*

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